THE ROLE OF POST-TRANSCRIPTIONAL MODIFICATIONS OF NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNITS ON THE TOXICITY OF SPINOSAD AND IMIDACLOPRID

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THE ROLE OF POST-TRANSCRIPTIONAL MODIFICATIONS OF NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNITS ON THE TOXICITY OF SPINOSAD AND IMIDACLOPRID

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ABSTRACT

Spinosad and imidacloprid are two of the most widely used insecticides. Both of these compounds act at the nicotinic acetylcholine receptor through mechanisms unique to each insecticide. High levels of resistance have been reported from a number of important agricultural and economic pests across the globe, often within a few years after the introduction of these insecticides.

Studies with laboratory created strains of *Drosophila melanogaster* indicate spinosad targets nicotinic acetylcholine receptors that contain the *Dα6* subunit. In an effort to validate these laboratory findings, I sequenced the *Pxylα6* subunit from the field collected Pearl-Sel strain of diamondback moth, *Plutella xylostella*, which has more than 18,000-fold resistance to spinosad. The *Pxylα6* subunit in Pearl-Sel possesses numerous premature stop codons that are unseen in two other spinosad susceptible strains. These truncated transcripts are genetically associated with spinosad resistance through the use of the F_2 backcross-bioassay method.

I chose to utilize RNAi in the red flour beetle, *Tribolium castaneum*, to systematically investigate the role of other nicotinic acetylcholine receptor subunits on toxicity of spinosad because RNAi is very robust in this species. I cloned of all 12 nAChR subunits in *T. castaneum* to use as templates for the production of dsRNA to use in RNAi. Sequencing these transcripts revealed a diverse array of posttranscriptional modifications such as alternative and cassette exon

use, intron retention, intron 3′ splice site variations, and a vast number of alleles. I used this information to design effective RNAi for the *Tcas*α*6* because my work on *P. xylostella*, and other work on *D. melanogaster* indicate that α*6* null mutants are resistant to spinosad.

RNAi was induced by injecting double stranded RNA for *Tcas*α*6* into pupae of *T. castaneum*. Silencing of *Tcas*α*6* produced no change in spinosad LC_{50} values despite a reduction in the expression of *Tcas*α*6*. To confirm this result, RNAi against the *D*α*6* subunit of *D. melanogaster* was performed using the Gal4-UAS system. There was no change in spinosad sensitivity in flies due to *D*α*6* silencing despite a significant reduction in *D*α*6* expression. These results indicate that RNAi against nicotinic acetylcholine receptors is not a feasible system to study the effect of specific subunits on insecticide sensitivity due to the large differences in the expression of nicotinic acetylcholine receptors and the RNAi machinery.

The Gal4-UAS system was utilized to silence the expression of Adenosine Deaminase Acting on RNA (ADAR) in different tissues of *D. melanogaster*. I chose this approach because it has been demonstrated that the Gal4-UAS system is effective at reducing the expression of ADAR, the level of ADAR expression is similar to the expression level of the RNAi machinery, and A-to-I RNA editing may be a factor in insecticide resistance. These ADAR-deficient flies were subject to spinosad and imidacloprid bioassays. Ubiquitous reduction in ADAR resulted in decreased spinosad insensitivity, while reduction in ADAR in cholinergic neurons and muscle increased spinosad insensitivity. Reduction of ADAR expression in cholinergic neurons, muscle, and glia increased imidacloprid insensitivity. These results indicate that editing is an important factor in insecticide insensitivity and the effect of editing is not spatially homogenous in the fly.

I used the peak height ratio method to estimate the frequency of A-to-I RNA editing to ensure the rate of editing was reduced via the Gal4-UAS system. The use of an antisense primer

showed very accurate and precise measurements of A-to-I RNA editing based on known editing rates. The accuracy and precision was consistent across different editing sites and expected editing frequencies. This method is more cost and time effective in comparison to other contemporary methods.

These results provide valuable insight into understanding and managing insecticide resistance. Firstly, they validate the use of a model organism to predict resistance in the instance of spinosad resistance. Secondly, they suggest that RNAi of nAChRs is not a suitable technique to evaluate target sites of spinosad and imidacloprid. Thirdly, A-to-I RNA editing affects the toxicity of spinosad and imidacloprid that varies depending on the tissues where it is expressed. These results will be of utmost importance in studies on population genetics, physiology, neurobiology, and mechanisms of insecticide resistance.

BIOGRAPHICAL SKETCH

Frank was born on 27-September-1980 in Wilkes-Barre, PA to Frank and Jane Rinkevich. His childhood was filled with many trips in the outdoors as part of annual camping trips with his family across the eastern United States, fishing the many lakes and rivers around his hometown with his grandfather, or simply building tree houses in the forests and collecting insects, snakes, frogs and turtles with his cousins and friends from the neighborhood. He was especially fond of the annual fishing trip with his grandfather, uncles, cousins, and friends where many stories were told and memories were made. These forays into the outdoors developed Frank's appreciation for nature and sparked an interest in biology and human health. Frank excelled in high school courses, but did not feel intellectually challenged. He spent most of his time writing essays, reading books, magazines and exploring the world around him.

After graduation from High School in 1998, Frank enrolled at Millersville University in Pennsylvania. Thankfully, this school provided an excellent opportunity to indulge in intellectual curiosity. Frank initially was attracted to the Marine Biology program, but after taking an entomology course with Dr. John Wallace, he switched his major to Environmental Studies to work with John in a more individual basis. Together, John and Frank performed experiments on the effect of stream restoration on populations of insects and fish, the role of insecticide exposure on fetal pig decomposition, and wrote an article for the Encyclopedia of Entomology on the history of fly fishing and entomology. This experience with Dr. Wallace encouraged Frank to pursue graduate studies in the field of entomology.

Dr. Jeff Scott welcomed Frank into his lab in the fall of 2002. Frank initially worked on *in vitro* expression of CYP6D1. As an auxiliary project, Frank began working with Dr. Li Zhang on the frequency of the pyrethroid resistance alleles for *Vssc1* and CYP6D1 in house fly

populations collected by Dr. Ronda Hamm as part of her thesis. This project showed that each location has its own combination of resistance alleles for both genes and it was the first to describe the full length cDNA for *kdr-his* and its role in permethrin resistance. Follow up studies have shown that the frequency of these alleles increase over the course of a field season through selection by pyrethroids, but then decline over the winter, indicating a fitness cost to resistant alleles. These studies on the population genetics of pyrethroid resistance alleles would become the topic of Frank's Master's thesis. He then left Cornell to take a position at a private company.

Frank was employed as a Technical Specialist at Home Paramount Pest Control in Bel Aire, MD. There, he was responsible for training employees, enforcing quality assurance and developing treatment protocols as a member of the technical department. This experience was highly influential in developing an effective and multifaceted teaching style. Frank yearned for opportunities for intellectual curiosity and professional development, so he left Home Paramount in the summer of 2006 to pursue other interests.

Frank earned a position as an instructor for introductory biology at Harford Community College in Bel Aire, MD. He taught three sections of lecture and lab where his passion for teaching was reignited. The students and administration he interacted with provided a high level of satisfaction with this position. However, after much thought and many tough decisions, he decided to reenroll at Cornell to return to work with Jeff Scott for his Ph.D. in the fall of 2007.

Upon returning to Ithaca, Frank decided to work on the role of nicotinic acetylcholine receptor subunits on insecticide resistance. He described the transcriptional diversity of nicotinic acetylcholine receptor subunits in the red flour beetle. Frank reported the first mechanism of spinosad resistance in a field evolved pest in diamondback moth. His final major paper focused on the role of A-to-I RNA editing on sensitivity of spinosad and imidacloprid in *Drosophila.*

Additionally, he wrote papers on allele competition studies of pyrethroid resistance alleles for *Vssc1* and CYP6D1 in house flies and an improved method for estimating the extent of RNA editing. Frank mentored an undergraduate student, Cathy Su, on her honors' thesis concerning alleles of *Vssc1* in the Colorado potato beetle. He also participated in studies on the frequency of pyrethroid resistance alleles in the house fly and cloning and sequencing the α6 subunit from western flower thrips. Frank served as Jugatae President from 2008-09 and Treasurer from 2010-11. He was also a participant in the annual Insectapalooza Entomology Open House by organizing a sales booth to raise Jugatae funds, demonstrations of neurophysiology, and advertising and promotions.

Frank hopes to earn a postdoctoral research appointment with Dr. Ke Dong at Michigan State University to study the role of A-to-I RNA editing on sodium channel electrophysiology in *Drosophila* or with Dr. Josh Rosenthal at University of Puerto Rico at Mayaguez to study the role of temperature on the rate of A-to-I RNA editing in *Drosophila*.

For my parents, Frank and Jane Rinkevich. Simply the best in the world.

And, for my Woobie.

ACKNOWLEDGEMENTS

I owe an immeasurable amount of gratitude to Dr. Jeff Scott for all he has done for me during my M.S. and Ph.D. He allowed me to return to his lab after I left prematurely after my M.S. in 2005. I was honoured that he provided me with a Sarkaria Fellowship for the first year of the program, which gave me the time and resources to start experiments immediately upon my return. Jeff found the time to meet with me to discuss projects, presentations and professional development despite being heavily burdened with the responsibilities as the Chair of Entomology during an extraordinary time for university administrators. I was very pleased with the number of papers that we wrote and other projects that I participated. Jeff also encouraged me to attend meetings other than the traditional Entomology Society of America's annual meeting to challenge my presentation skills and allow me to meet with other colleagues. Jeff, together with my other committee members, Drs. Cole Gilbert and Ron Harris-Warrick, were extremely helpful at developing my critical thinking ability outside the view of insecticide resistance, challenging my intellectual capacity, and assisting me with career advice. I am also thankful for their comments on my dissertation and their ability to accommodate my urgent deadlines. I am also grateful to Dr. Bruce Johnson for being a teaching assistant for his Principles of Neurophysiology course. I feel my interactions with Bruce were extremely beneficial to developing my teaching philosophy and honing my lecturing technique. I am also thankful to the above people for their patience and their fast turnaround on letters of recommendation needed for imminent deadlines.

I was fortunate to work with some very talented members of the Scott lab. My fellow graduate students Drs. Ronda Hamm, Melissa Hardstone, and George Lin provided excellent advice on what to expect as I progress though my program, how to prepare for my qualifying

exams, as well as collaborating on research projects. Our lab manager, Cheryl Leichter, has been a constant source of expertise on insect rearing and bioassays, as well as being a sounding board for all my bad jokes and stories. I have shared lab space with a number of talented undergraduate researchers, especially Cathy Su, Tomas Lazo, and Sarah Harris, all of whom have gone on to prestigious Graduate and Medical schools. I am thankful for working with Brandon Loveall and Hannah Kim from David Dietcher's lab as well as Dr. Brian Lazzaro for their help with *Drosophila* rearing and genetics. I also would like to thank John Diaz for transporting samples of diamondback moth from Geneva to Ithaca.

The graduate student body was immensely pleasurable to interact with. I felt that we were very productive as a group and much more organized than my experience during my Masters'. I was lucky to live and work with two other entomology graduate students, Calum Russel and Erik Smith. Their friendship was invaluable for dealing with issues with administration, course work, and experiments. Although, we were in different departments, I have made a life-long friend in Mike French. We both enjoy recapping, strategizing, philosophizing, watching and participating in sports, especially with our summer softball team, The Upper Deckers, and our beer pong team, Come at me Bro! in which dominated our veterinary, law and business school opponents. I will never forget going to New York with Mike to watch Yankees games, especially Derek Jeter's 3000^{th} hit on 9-July-2011, and going all over the city like we owned it. Our Thru-Way Challenge food road trip is the stuff of legends!

I would not have been able to resume graduate studies without the support of my best friend and partner, Kim Snyder. I was sustained and soothed by our unspoken understanding of each other's thoughts, pleasures and goals. She has provided a constant source of fun and satisfaction over the duration of my studies. I never thought I would meet someone that I would

fall head over heels in love with until I met Kim. Although we have had to endure the challenges and pitfalls of a long distance relationship, we have stayed strong for longer than I would have ever thought. She is the most important person in my life outside of my family. I can't wait to write my life story with this woman as my co-author.

Finally, the most important people I would like to acknowledge for my completion of my Ph.D., my parents, Frank and Jane Rinkevich. As I have grown older, the more I appreciate the sacrifices they have made to provide for my sister and I. They worked long hours, denied themselves expensive wares or vacations, and made our happiness and development their priority. I have learned invaluable lessons about loyalty, frugality, diplomacy, reliability, and empathy from the examples they have set. They have always encouraged my sister and me to pursue our interests without any restrictions and their entire support. I am fortunate that they still participate in activities that I enjoy, such as attending sporting events and travelling to different cities. They are not only my parents but two of my best friends. I hope that I can provide a comparable level of support and entertainment to my family in the future.

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1.1 Nicotinic Acetylcholine Receptors

1.1.1 Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of the ligand-gated ion-channel superfamily of neural receptors which include serotonin, γ-amino butyric acid (GABA), and glycine receptors. All ligand-gated ion-channels possess a cys-loop motif, $C(X_{13})C$, in their extracellular, ligand binding domains. They feature four membrane spanning domains, of which the second domain (TM2) forms the pore of channel. Ligand-gated ion-channel subunits form homo or heteropentameric complexes (Figure 1). It is likely that the first nAChRs were homopentamers (Ortells and Lunt, 1995).

Nicotinic acetylcholine receptors were first purified from the electric organ of the electric ray (*Torpedo sp.*) using α-bungarotoxin as a high affinity purification ligand (Weber and Changeux, 1972). The first few residues of these purified proteins from *Torpedo marmorata* and *T. californica* were sequenced and found to be homologous proteins (Raftery et al., 1980), which facilitated the cloning of the cDNAs for these proteins (Noda et al., 1982). The deduced amino acid sequences from these cDNAs allowed for a more precise definition of many aspects of nAChRs mentioned above (Ortells and Lunt, 1995).

1.1.2 Composition and Structure

nAChRs can be homopentamers of α subunits or heteropentamers of both α and non-α subunits. All α subunits possess a characteristic YxCC amino acid motif found in loop C of the extracellular N-terminal domain whereas β subunits (and other non-α subunits such as γ, δ or ε)

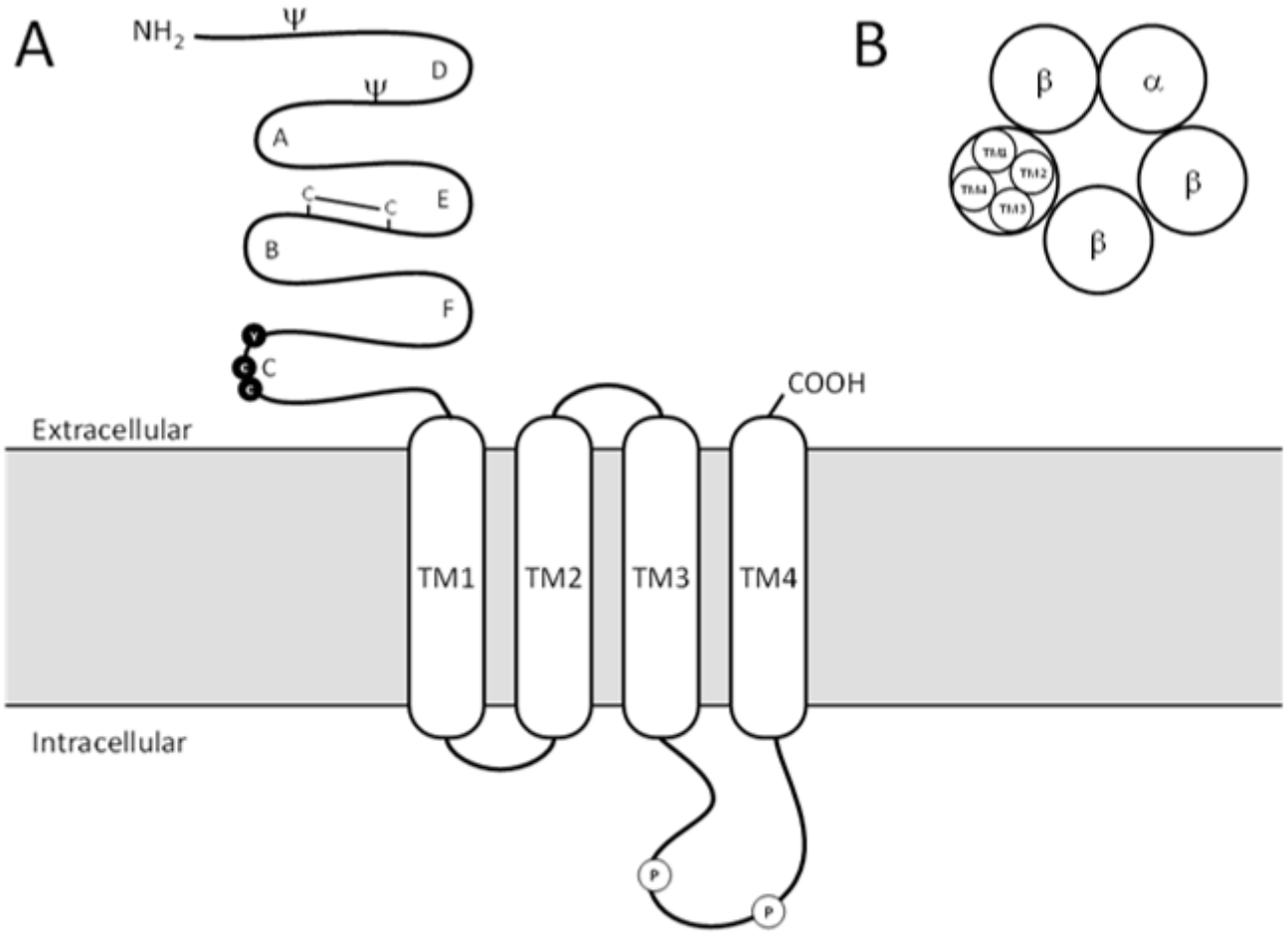

Figure 1.1 Structural diagram of the nicotinic acetylcholine receptor subunit and a receptor complex. A) Extracellular loops A-F are labeled near the N-terminus. Transmembrane domains (TM) are indicated by white ovals. *N*-linked glycosylation sites are marked with a ψ. The disulphide bond formed by the cys-loop motif is indicated between loops E and B. The YXCC α subunit signature is indicated by black circles. Phosphorylation sites between TM3 and 4 are indicated by white circles. B) The receptor complex represents a heteropentamer of α and β subunits and the orientation of the TM domains within each subunit.

subunits lack this feature. There are three major structural features of each subunit: 1) the ligand-binding/facilitating N-terminal domain with sequential loops D, A, E, B, F, and C and the distinctive C(X_{13})C amino acid signature for ligand-gated ion-channels is found between loops E and B, 2) four transmembrane domains (TM) that form the cationic pore, and 3) the intracellular linker between TM 3 and 4 (Figure 1). The ligand-binding domain and intracellular linker are both subject to post translational modification to create pharmacologically unique receptors.

1.1.3 Ligand Binding

The α subunits are required for acetylcholine (ACh) and other agonist binding, but this interaction requires adjacent subunits (α or non-α). Photoaffinity labeling with competitive agonists such as benzenediazonium fluoroborate, d-tubocurarine, α-bungarotoxin and nicotine with combinations of α and non-α subunits showed that most of the binding occurs on the α subunit, but the non-α subunits were also labeled to a lower degree (Corringer et al., 2000). Therefore the α subunits carry the "principle component" of the agonist binding site while non-α subunits carry the "complimentary component". The "principle component" of α subunits include aromatic resides W86, Y93, W149, Y151, Y190 and Y198, and nucleophilic residues C192 and C193 on loops A, B and C. The "complimentary component" is composed of W55, W57, Y111, R113, D165, D180 and E182 on loop D, E and F of non-α subunits (Corringer et al., 1995). Interestingly, the "complimentary components" are also found on α subunits that are able to form homopentamers (Corringer et al., 1995, Galzi et al., 1991). The prevalence of aromatic residues at the binding site suggests the electrons found in the side chains provide a negative electrostatic surface that is used to stabilize interactions with quaternary ammonium of ACh (Sine et al., 1994, Dougherty and Stauffer, 1990). The tryptophan residue at position 149 of the α

subunit (αTrp-149) mediates interaction with the cationic portion of acetylcholine. This finding was elucidated through an elegant set of experiments using nonsense codon suppression to incorporate unnatural tryptophan derivatives into the α subunit. These modified tryptophan residues had varying levels of electronegativity and steric bulkiness. The effect of these groups was investigated using expression in *Xenopus* oocytes. There was a strong negative linear correlation between the strength of the cation–π binding and EC_{50} of ACh. This interaction is specific for tryptophan as substituting this residue with phenylalanine increases the EC_{50} 100-fold. This change in EC_{50} is due to the fact that the aromatic side group of phenylalanine aligns with the 5-membered ring of tryptophan and is poorly positioned for binding interactions. Interestingly, incorporating Tyr-O3Q which places the quaternary ammonium of the unnatural amino acid at the same position where acetylcholine binds produces a constitutively active channel (Zhong et al., 1998). These lines of evidence are supported by the observation that the quaternary ammonium portion of acetylcholine interacts in a similar manner to tryptophan 84 in acetylcholine esterase (Sussman et al., 1991).

1.1.4 Cationic Pore

The site of action of noncompetitive blockers suggests they bind to residues in TM2 (reviewed extensively in (Changeaux and Edelstein, 2005)). The nAChR blockers tetracain, procain, dimethisoquin, and others, were evaluated for inhibition of [^{3}H] α-bungarotoxin binding and compared to known competitive agonists such as decamethonium, carbamylcholine, and flaxedil. The effect of blockers was seen at concentrations two orders of magnitude higher than competitive agonists. This suggests the site of action is at a site distinct from the competitive agonist binding site (Weber and Changeux, 1972). Chlorpromazine is a noncompetitive blocker.

Membrane bound receptors from *Torpedo marmorata* photolabeled with [^{3}H] chlorpromazine and then subject to protein fractionation and sequencing, showed that serine 262 of the δ subunit was labeled by [^{3}H] chlorpromazine (Giraudat et al., 1986). The α and β subunits were labeled at homologous positions (Giraudat et al., 1987, Hucho et al., 1986). These residues are located on the TM2 segment of each subunit.

TM2 contains amino acid residues that are critical to proper flow of ions (Imoto et al., 1988). TM2 is composed of a loop and α-helix that forms the pore's geometry and determines the ions that may pass. The TM2 loop forms the narrowest portion of the channel which limits the size of permeable cations, selects Ca^{2+} as the only divalent cation able to move through the pore, and prevents the flow of anions. The E237 residue in the TM2 loop has a major influence on ion selectivity (Imoto et al., 1988, Bertrand et al., 1993, Galzi et al., 1992). The TM2 α-helix of the pore forms a funnel that allows for fully hydrated ions to enter the extracellular opening of the pore, but they become less hydrated as they travel through the narrowing pore. The progressive dehydration of the ions makes the charge filter of the loop region of the channel more selective and efficient (Corringer et al., 2000). The TM2 segment is separated from the other TMs by a water filled space that allows TM2 to move when the channel is activated (Unwin, 1995). The binding of ACh to the ligand binding site in the extracellular domain overcomes the energy of interaction of the α subunit with its neighboring non α subunit (Unwin et al., 2002). This relaxes the conformation of the protein and causes TM2 to rotate 15^o on its axis. This rotation of TM2 causes the channel to widen and allows ions to flow through the channel (Unwin, 1995, Unwin et al., 2002, Unwin, 2003).

1.1.5 TM3-TM4 Intracellular Linker

The intracellular linker between TM3 and TM4 (Figure 1.1) is important for a variety of important functions. Intracellular trafficking and membrane placement are controlled by conserved hydrophobic residues of this linker (Millar and Harkness, 2008). The linker is important for cell surface expression, protein folding and functional expression. Chimeric proteins were created by replacing the N-terminus of rat 5-HT_{3A} with the N-terminus of rat nAChR α7 subunit. A series of mutants was created by exchanging the 5-HT_{3A} intracellular linker with the one from rat α1-10 and β1-4. The intracellular linker from rat α2, 3, 5, 6, 9, 10 and β1-4 showed lower cell surface expression compared to the intracellular linker from 5-HT_{3A} as measured by reduced [^{125}I] αBTX binding. Those same intracellular linkers also interfered with intracellular folding as demonstrated by reduced [^{3}H] MLA binding. Only constructs with loops from α3, 7, 8, or 10 created functional channels when expressed in tsA201 human embryonic kidney cells (Kracun et al., 2008).

Highly conserved leucines and phenylalanines were identified in an alignment of the first thirty amino acids of the cytosolic loops of rat α1-10 and β1-4. These hydrophobic residues were mutated to alanine in various combinations using α4 and β2 subunits expressed with complimentary subunits in tsA201 cells. These mutations dramatically reduced cell surface expression and epibatidine binding capacity as reflected by the higher degradation rate and/or lower folding efficiency of the mutant subunits. Wild-type β2 co-localizes with the Golgi marker, giantin, while the $\beta2^{L351,\ 357,\ 358A}$ mutant completely abolishes this association, suggesting that β2 is necessary for proper export of the receptor from the ER. A similar, but weaker effect was seen with the $\alpha4^{L351,\ 357,\ 358A}$ mutant (Ren et al., 2005).

1.1.6 Post-translational Modifications of nAChRs

The function of nAChRs can be modulated by phosphorylation and *N*-glycosylation (Thany et al., 2006, Nishizaki, 2003). Phosphorylation by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), protein tyrosine kinases (PTK), and calmodulin-dependent protein kinases (CAM-K) are at serine and tyrosine residues on the intracellular loop between TM3 and TM4 (Swope et al., 1992). Phosphorylation by kinases appears to be subunit specific. PKA is specific to α Ser-357, γ Ser-353 and δ Ser-361 (Huganir and Greengard, 1983, Yee and Huganir, 1987), PKC phosphorylates δ Ser-362, 377, and 379 (Safran et al., 1987), and PTK phosphorylates β Tyr-355, γ Tyr-364 and δ Tyr-372 and (Huganir et al., 1984, Wagner et al., 1991). Postsynaptic membrane fractions enriched for nicotinic acetylcholine receptors were phosphorylated *in vitro* at the γ and δ subunits using purified CAM-K.

ACh induced ion flow is not influenced by phosphorylation status. However, phosphorylated receptors desensitized 7-8 times faster than unphosphorylated receptors. The degree of phosphorylation for a population of receptors affects the rate of desensitization. When treated with a ratio of 0.6 mol phosphates per mol of subunit, 82% of receptors desensitized rapidly while a ratio of 0.4 mol phosphate per mol of subunit caused 64% of receptors to desensitize rapidly. The rate of desensitization is dependent on the concentration of ACh (Huganir et al., 1986). PTK affects receptor desensitization. Purified nAChRs from *T. californica* were dephosphorylated by alkaline phosphatase to stoichiometries between 0.6-2.7 mol phosphates per mol of receptor. Single channel properties were recorded with inside-out patches of reconstituted membrane with giga-seal patch-recording techniques. The time constant for the fast desensitization rate decreased with increasing phosphorylation.

N-linked glycosylation affects receptor function, assembly and trafficking. Rat α7 is able to form homopentamers and is glycosylated *in vivo* at N46, N90 and N133 on the N-terminal ligand-binding domain (Chen et al., 1998). Site-directed mutagenesis was used to generate N46A, N90A, or N133A mutants that would disrupt the *N*-glycosylation NXS/T consensus sequence. The N46A, N90A and N133A mutants showed a 3-fold increase, 1.5-fold decrease, and no response, respectively, in peak current compared to the wild-type. To demonstrate these changes were due to deglycosylation and not structural changes in the protein, the serines of the three NXS/T *N*-linked glycosylation consensus sequences mentioned above were mutated to alanine. Mutating the consensus sequence serines to alanine produced the same changes in peak current as in the aspargine mutants as mentioned above, suggesting that the loss of glycosylation causes the change in peak current. The expression of α-BTX binding sites for the N46A, N90A, or N133A mutants are increased 3-fold, unchanged, and reduced 3-fold, respectively. These results suggest glycosylation at N46 and N133 have critical roles in functional expression of α7 homopentamers (Chen et al., 1998). Glycosylation is not important for cell surface expression because there is no change in [^{125}I] streptavidin binding for N46A, N90A, or N133A mutants. It is likely glycosylation is important for proper receptor folding and stabilization (Chen et al., 1998). Site directed mutagenesis was used to create *T. californica* mutant subunits. Native *T. californica* receptors have a $\alpha_2\beta\gamma\delta$ stoichiometry. The whole cell current decay time for receptors with δN143D decayed slightly, but significantly longer than the controls. The βT143A and γS143A receptors decayed an order of magnitude longer than the controls, suggesting the serine or threonine of the NXS/T *N*-linked glycosylation consensus sequence is more critical to desensitization than the asparagine. Single channel recordings showed the βN141D, βT143A, and γS143A mutants desensitized slower than wild-type, suggesting glycosylation sites on β and

γ subunits were responsible for desensitization. The βN141D and βT143A mutants had accelerated decays in the fast component of desensitization, but also significantly longer decays of the slow component. The βN141D mutant had a significantly lower single channel conductance than the wild-type but no change in mean open time. These results suggest the βN141 residue is a critically important *N*-linked glycosylation site that affects many aspects of receptor function. Interestingly, binding of lectin concanalvin A to the sugar groups has a similar effect as the mutations mentioned above. Therefore, the sugar groups may act to enhance the rate of desensitization by acting as a lid to block the pore of the channel (Nishizaki, 2003). Upon treatment with the glucosidase inhibitors castanospermine or tunicamycin, the amount of cell surface expression of nAChRs as measured by [^{125}I]Btx is reduced 75% and 100%, respectively. Neither castanospermine nor tunicamycin had an effect on subunit synthesis, suggesting that *N*-linked glycosylation is important for proper nAChR assembly (Wanamaker and Green, 2005).

1.1.7 Insect nAChRs

1.1.7.1 Genomes and Gene Families

Insects possess a diverse array of nAChR subunits in terms of number and post-transcriptional modifications. In insects, there are 10 subunit genes in *Anopheles gambiae* (Jones et al., 2005), 11 in *Apis mellifera* (Jones et al., 2006), 12 in *Bombyx mori* (Shao et al., 2007), 10 in *Drosophila melanogaster* (Sattelle et al., 2005), 12 in *Tribolium castaneum* (Jones et al., 2007) and 16 in *Nasonia vitripennis* (Jones et al., 2010). There are no γ, δ, or ε subunits in insects. Most insect α subunits share more than 70% similarity in the deduced protein sequences between species. The α5 subunit is the least similar across species. The β1 subunit is orthologous across species but the β2 subunit in *D. melanogaster* is orthologous to the α8 subunit in *T.*

castaneum, *A. gambiae*, *A. mellifera*, and *B. mori*. *Tcasα11* is also included in this group. Individual species may possess divergent subunits that have no clear ortholog is any other species (less than 35% similarity). This includes the α subunits *Agamα9*, *Amelα9*, *Amelβ2*, *Bmα9*, *β2* and *β3*, *Dβ3*, *Tcasα9* and *10*. The numbering of nAChR subunits relies on similarity to other subunits, but this convention is vague and misleading when applied to divergent subunits. Subunit nomenclature is in need of standardization and clarification (Lukas et al., 1999). Insect nAChR subunit gene transcripts are subject to post-transcriptional modifications to generate a diverse pool of transcripts from a limited number of genes. More than 30,000 *Dα6* isoforms are theoretically possible due to combinations of alterative exon use and A-to-I RNA editing (Grauso et al., 2002).

1.1.7.2 Alternative Splicing

Alternative exon use is conserved across insects and appears to be largely restricted to the *α4* and *α6* subunits. This observation suggests that alternative exon use in nAChR subunit transcripts is as old as the existence of holometabolous insects (Jin et al., 2007). The only example of a non *α4* and *α6* subunit that exhibits alternative exon use is *Bma8* (Shao et al., 2007). Exon 4 of the *α4* subunit undergoes optional exon use between 2 exons, 4 and 4′. These alternative exons share more than 90% similarity across species. Alternative exon use is much more complex in the *α6* subunit. Exons 3 and 8 contain 2 and 3 alternative exons, respectively, but this can vary between species. The ancestral condition is thought to be exon 3a and 3 versions of exon 8 (8a, 8b and 8c). Exon 3b is found in the lineages of Coleoptera (*T. castaneum*), Lepidoptera (*B. mori*), and Diptera (*D. melanogaster*, *A. gambiae* and *Musca domestica*), but not included in Hymenoptera (*A. mellifera*). Exon 8a is absent in *B. mori* and *A.*

gambiae, but retained in *D. melanogaster*, *M. domestica* and *T. castaneum*. Exon 8b shares the highest amino acid conservation across species (Jin et al., 2007). This conservation is thought to be a reason that it is the most frequently used exon 8 from those species. While exon 8c is genomically encoded by all species, it is included in less than 3% of transcripts, and it shares the lowest amount of amino acid conservation relative to 8a and 8b (Jin et al., 2007). Transcripts possessing both exons 3a and 3b have been reported in *Musca domestica* (Gao et al., 2007c), *D. melanogaster* (Grauso et al., 2002), *B. mori* (Shao et al., 2007, Jin et al., 2007) and *T. castaneum* (Rinkevich and Scott, 2009, Jones and Sattelle, 2007). Transcripts with exons 3a and 3b become more common from embryo to adult in *B. mori* (Jin et al., 2007). Having both exons 3a and 3b would duplicate the acetylcholine binding loop D. It is unknown if subunits with repeated ligand binding loops would alter ACh binding or not. The only example of inclusion of both exon 8a and 8b is in isoform V of *Dα6* (Grauso et al., 2002), despite intense research in *T. castaneum* (Rinkevich and Scott, 2009) and *M. domestica* (Gao et al., 2007c). There are two *α6* alternative exon isoforms in *B. mori* (Shao et al., 2007), 6 in *D. melanogaster* (Grauso et al., 2002), 10 in *M. domestica* (Gao et al., 2007c) and 18 isoforms in *Tcasα6* (Rinkevich and Scott, 2009).

1.1.7.3 Cassette Exons

Optional or cassette exons are designated as exons that can be omitted from transcripts. Most often, optional exons lead to the introduction of premature stop codons. In some cases the transcript remains in frame, but proteins produced from these transcripts would have structural deficits. It is unclear if proteins created by transcripts with optional exons posses any function, but the wide spread prevalence and conservation of such transcripts suggest a possible role for these proteins. Exons 2 and 4 of *Dα4* can be optional exons. Transcripts that lack exon 2 are

missing loop D, while omission of exon 4 creates a premature stop codon (Sattelle et al., 2005). Exon 4 is also skipped in *Agamα4* (Jones et al., 2005) and *Amelα4* (Jones et al., 2006), introducing premature stop codons in both cases. In *Bmα4*, exons 6-8 are excluded from a transcript but do not produce a frameshift or premature stop codon. These transcripts, however, would produce proteins that lack loop F and TM1-3. Omission of exon 5 of *Dα5* or exon 4 of *Bmα5* introduce premature stop codons (Sattelle et al., 2005). Exons 3-8 of *Tcasα6* are optional exons which generate 13 different isoforms which have premature stop codons or structural insufficiencies (Rinkevich and Scott, 2009). Other examples of option exon use are exons 5-7 of *Mdα6* (Gao et al., 2007c) and exons 2 and 3 of *Agβ1* (Li and Han, 2005). Although *Dα4*, *Agamα4*, *Amelα4*, *Bmα4*, *Tcasα6*, and *Mdα6* are alternatively spliced, *Dα5*, *Bmα5*, *Agβ1* are not. Thus, alternative exon use is not a requirement for optional exon use.

1.1.7.4 Retained Introns

Retained introns that create premature stop codons have been reported from many insects. Unspliced introns were reported from *Agamα7* (Jones et al., 2005), *Amelα3* and *α7*, *Dα7* (Grauso et al., 2002), (Jones et al., 2006), *Mdα6* isoform VIII (Gao et al., 2007c), and *Tcasα3, 4, 9, 10,* and *β1* (Rinkevich and Scott, 2009). All unspliced introns introduce a premature stop codon shortly after the introduction of the intron into the open reading frame except in *Agamα7*, which remains in frame for an additional 86 amino acids before the stop codon. Intron 9 in *Mdα6* isoform VIII was only 59 bp in length. Intron 5 was found unspliced in transcripts of *Agamα7*, *Amelα7*, and *Dα7* with lengths of 7292, 3666, and 62 bp respectively. Thus, it appears intron length may be a species specific determinant to leave introns unspliced from mature transcripts. Unspliced intron 9 in *Tcasα3* and *Amelα3* and intron 6 of *Tcasα11* produce transcripts that are

truncated just after TM3 while unspliced intron 7 of *Tcasα4* is truncated halfway through TM3. Similarly truncated transcripts of *Dα1* abolished ACh-mediated inward currents (Schulz et al., 2000). EMS mutagenesis that produced truncated *Dβ2* after TM3 created a strain of *D. melanogaster* that was resistant to imidacloprid and other neonicotinoids (Perry et al., 2008).

1.1.7.5 Intron Splice Site Variants

Variants in the 3′ splice site of introns were found in relatively few subunits. *Amelα3* has a short and long variant. In the long variant, *Amelα3L*, 39 bp are added that code for an extra 13 amino acids in the intracellular region between TM3 and TM4. These additional residues add two putative phosphorylation sites to an area of the protein involved in receptor function via phosphorylation (Section 1.1.6). An alternative donor site of intron 10 of *Mdα5* deletes 15 bases that removes a YLENL motif in the intracellular linker (Gao et al., 2007b). Splice variants at the 3′ splice sites of introns were observed in *Tcasα2*, *3*, *6*, and *10*. These splice variants introduce a premature stop codon. In *Tcasα2*, intron 3 is spliced out 5 bp downstream of the typical 3′–AG intron boundary. Intron 4 of *Tcasα3* was spliced out 10 bp upstream. Introns 5 and 7 in *Tcasα6* were spliced out 179 and 91 bases upstream, respectively. Intron 5 of *Tcasα3* is spliced out 13 bp downstream (Chapter 4.3.4 (Rinkevich and Scott, 2009).

Many nAChR subunit transcripts undergo a diverse array of modified splicing events that generates premature stop codons. It is unknown if these transcripts are subject to nonsense mediated decay (Chang *et al*., 2007). Although no physiological studies have identified a role for these truncated transcripts in insects, it has been suggested they code for proteins that may moderate synaptic events in a similar manner to ACh-binding protein in *Lymnaea stagnalis* (Sattelle et al., 2005) or α7 in mice (Saragoza et al., 2003).

1.1.7.6 A-to-I RNA Editing

A-to-I RNA editing can generate transcript diversity that can result in proteins with altered function or distribution that may affect behavior and life history traits in many animals. A-to-I RNA editing is found in all animals except the trematode. The widespread conservation of this pathway is thought to be a viral defense mechanism (Bass and Weintraub, 1988, Bass, 2002). A-to-I RNA editing is performed by adenosine deaminases that act on RNA (ADARs) that bind to double stranded pre-mRNAs and convert adenosine to inosine which is recognized by translational machinery of the ribosome as a guanosine. These modifications can be seen in the protein coding and non-coding sequences, introns, 5′ and 3′ untranslated regions of the pre-mRNA which may result in changes in the amino acid sequences, splice sites or levels of transcripts (Bass, 2002). Two versions of ADAR (i.e. ADAR1 and ADAR2) is the ancestral condition and the loss of ADAR1 is unique to insects and crustaceans. Insect Adar is an ortholog of ADAR2 and shares conserved function (Keegan et al., 2011).

The transcripts of many ligand-gated or voltage-sensitive ion channels and G-protein coupled receptors are targets of A-to-I RNA editing (Bass, 2002, O'Connell, 1997, Paul and Bass, 1998, Hoopengardner et al., 2003). Genome wide studies in *D. melanogaster* have shown conserved wide-spread editing of these genes (Hoopengardner et al., 2003). RNA editing of these genes is focused on regions that code for important amino acids in the protein. In voltage-gated K^+, Na^+ and Ca^{2+} channels, residues involved in channel gating or inactivation are edited. Editing sites on nAChR or GABA receptor subunits occur in crucial areas in the ligand-binding domain and TM2 that forms the channel pore (Hoopengardner et al., 2003).

D. melanogaster dADAR$^-$ null mutants show no A-to-I RNA editing, developmental abnormalities, or changes in longevity, but adults show significant deficiencies in motor control

and mating that grow progressively worse with age. Male *dADAR*$^{-}$ null mutants do not initiate courtship displays and are not able to successfully mate. Adults jump and fly only when constantly harassed. Leg and wing tremors and persistent flexion become more frequent with age. At 30 days old, they exhibit circular walking. Most flies over 50 days old constantly have their wings upheld and show uncoordinated movements that result in long periods of prostration. Nervous system morphology is greatly affected by *dADAR*$^{-}$ null mutants. The photoreceptors are disorganized and extended longitudinally to the laminar layer. In 30 day old adults, massive lesions appear in the central brain, optic lobes and retina and become progressively more degenerate with age. These morphological changes coincide with behavioral deficits, but a causal relationship has not been determined (Palladino et al., 2000b).

Behavioral deficits were also seen in *D. melanogaster* adults that have *dADAR* activity reduced by using the Gal4-UAS system to express dsRNA against *dADAR* in various tissues. Flies that expressed dsRNA for *dADAR* did not fly in a vertical glass cylinder and did not exhibit diurnal activity patterns. RNAi against *dADAR* also produced temperature sensitive paralysis when warmed to 39^{o}C for 2m. Expression of behavioral deficits manifest in flies that had pan-neuronal expression of dsRNA for *dADAR* in excitable cell types such as neurons, muscle and glia. Smaller but still significant reductions in behavioral activity were seen when dsRNA for *dADAR* was expressed in the mushroom bodies (Jepson and Reenan, 2009).

Flies were engineered to be hypomorphic for *dADAR* by using ends out homologous recombination. Flies homozygous for this hypomorphic character had reduced levels of editing in many tissues. Similar to *dADAR* null flies, these *dADAR*^{hyp} flies had reduced diurnal activity. Male flies took longer to initiate courtship behaviors. The pattern of courtship song was altered in male *dADAR*^{hyp} flies which had fewer pulses, higher frequency, and a longer inter-pulse

period. The changes in courtship song were driven by reduced editing in *fruitless* neurons (Jepson et al., 2011).

RNA-editing is thought to act by compensating for G-to-A mutations to revert them back to G (Tian et al., 2008). More than 16 A-to-I editing sites have been identified in the α6 nAChR subunit from eight insects spanning more than 300 million years of evolution. Two sites are edited in all holometabolous insects such as *D. melanogaster*, *M. domestica*, *B. mori*, and *A. mellifera* besides *A. gambiae*, which does not possess any RNA editing ability (Jones et al., 2005). These editing sites are genomically encoded G in the more primitive hemimetabolous *Pediculus humanus*. The results of these editing sites create codons at those positions that reverts the derived amino acids asparagine and isoleucine to the primitive amino acid aspartic acid and methionine, respectively. Other editing sites in these insects are encoded genomically G in many instances. This evolutionary conservation of editing was used to predict a previously unknown editing site in *cacophony* of 12 *Drosophila* species. A site that is G in *A. gambiae*, *B. mori*, *D. grimshawi* and *virilis* is A in the evolutionarily more derived sophophora Drosophilids such as *D. melanogaster*. Transcripts of the derived flies undergoes RNA editing that reverts this codon for isoleucine to methionine (Tian et al., 2008).

1.1.8 ACh Binding Protein as a Model for nAChR Studies

Acetylcholine binding protein (AChBP) is released from glial cells in mollusks such as the great pond snail, *Lymnea stagnalis*, and the sea slug, *Aplysia californica*. AChBP modulates cholinergic synaptic transmission where high concentrations of AChBP suppress synaptic transmission (Sixma and Smit, 2003). AChBP is 210 residues long and shares 20-25% identity to the ligand binding domain of nAChRs. The α-subunit defining YxCC motif as well as the

$C(X_{13})C$ signature of cys-loop ligand-gated ion channels are present in AChBP (Smit et al., 2001, Sixma and Smit, 2003). When expressed in the yeast, *Pichia pastoris*, AChBP forms stable homopentamers much like α7-9 (Smit et al., 2001, McGehee, 1999). A comparison of the crystal structure of AChBP to the 4.6 Å electron microscopy information of the extracellular ligand binding domain nAChRs shows both proteins have similar architechture (Unwin et al., 2002). The AChBP is pharmacologically comparable to a homopentamers of nAChR α7 (Unwin et al., 2002). The structural and pharmacological similarities have allowed for relatively reliable ligand and toxin binding models for nAChRs based on AChBP to be created (Le Novere et al., 2002, Sine et al., 2002).

1.2 Importance of Insects

1.2.1 Impacts of Insect Pests

Insects can directly impact human health through disease transmission or indirectly by destroying food crops. The economic impact of damage to crops such as cotton and tobacco can be extensive and profound. Wood boring pests can cause extensive and dangerous structural deficiencies. Insect contamination of food stuffs can damage a manufacturer's reputations and lead to decreased sales. There is also a social stigma associated with insect infestations. As such, insects may negatively impact every aspect of the human condition and their influence will significantly increase as the global population grows exponentially (Pimentel et al., 1992).

Insects are responsible for some of the most devastating epidemics in human history. The bubonic plague that swept across Europe in the 14th century caused up to 60% mortality in some areas and killed an estimated 100M people across the globe. The "Black Death" was transmitted to humans by fleas that became infected with the bacterium, *Yersinia pestis*, by feeding on rats.

Malaria is a modern pandemic. More than 500M people globally are infected with the malaria parasite, *Plasmodium falciparum*, resulting in about 1-3M deaths annually. Malaria is transmitted person to person by *Anopheles* mosquitoes that preferentially feed on human blood. It is a prevalent tropical disease. There are relatively effective drugs to treat malaria but effective immunization remains elusive. Dengue fever shares a similar geographic distribution as malaria but is transmitted by the day feeding mosquitoes *Aedes aegypti* and, to a smaller degree, *A. albopictus*. More than 50M people are infected with dengue every year. Infection with multiple serotypes of dengue may result in highly deadly hemorrhagic fever. Emerging diseases in the United States such as West Nile fever, various form of encephalitis, and Lyme disease are caused by insect vectors.

Insects cause tens of billions of dollars worth of damage to global field crops and stored commodities. In the US, the armyworm, *Helicoverpa spp.*, diamondback moth, *Plutella xylostella*, and red flour beetle, *Tribolium castaneum*, each cause >$1B in crop damage (Throne et al., 2003, Talekar and Shelton, 1993). In some extreme cases of infestation where control measures are ineffective, entire fields are intentionally destroyed or abandoned by growers (Arbo et al., 1994, Perez et al., 2000, Reed and Pawar, 1981). Infestation of commodity crops can significantly reduce the storage life and nutrition quality(Scott, 1991).

Wood infesting pests such as termites and carpenter ants cause more than $2B in global economic losses in non-structural wood commodities (Verma et al., 2009). Residential homes are prime targets for wood destroying pests because there are more than 100M units, they are built in formerly forested areas, preventative treatments are rarely used, and sometimes built using haphazard construction practices (Mauldin, 1986). Control of Formosan termites in New Orleans costs more than $300M, annually (Suszkiw, 1998).

The fear of insects can have devastating psychological impacts. The irrational fear of imaginary insects is called delusory parasitosis. Individuals suffering from delusory parasitosis often report crawling, burrowing or itching sensations on various parts of their body. Skin lesions may be due to scratching or digging out the imaginary burrowing pests or use of hazardous chemicals to eliminate the infestation. Static electricity, household chemicals, environmental variables, mechanical irritants, medical and/or psychological conditions and a litany of medications may be responsible (Hinkle, 2000).

1.2.2 Importance of Insecticides

Mankind has sought practices to control insects due to the devastating impacts insects have on human life. Insecticide use dates back 4500 years. Sulfur was the first recorded insecticide. Other inorganic materials such as arsenic and lead and various plant extracts were utilized for modest and minimally effective insect control (Perry et al., 1998). The age of modern synthetic insecticides arose in the early 20th century with the discovery of the insecticidal properties of DDT. It has been heralded as a panacea for controlling insects affecting crops and human health (Wright et al., 1972). There are currently hundreds of insecticides in 9 major classes currently available for insect control (Nauen 2002). Annual global sales of insecticides exceed $25B and have a four-fold return on investment in terms of crop yield increases (Pimentel et al., 1992). In the US, $4B worth of more than 600 types of pesticide totaling >500,000 tons are used to control insects (Pimentel, 1991). However, these costs are offset by the negative impacts of pesticides on humans, livestock, and the environment. These losses total more than $8B per year (Pimentel et al., 1992). Therefore, newer, more effective pesticides with

high target specificity and minimal negative impacts on human and environmental health are highly desired.

1.3 Insecticides Acting at the nAChR

1.3.1 Spinosad

1.3.1.1 Introduction

A sample of fermentation broth of an isolated culture from a defunct rum distillery in the Caribbean, later named A83543, showed positive insecticidal activity against larvae of the mosquito, *Aedes aegypti* and southern armyworm, *Spodoptera eridana*. A83543 did not show activity toward spider mite (*Tetranychus urticae*), aphids (*Aphis gossypi*), corn rootworms (*Diabrotica undecimpunctata howardi*), or nematodes (*Melodiogyne arenaria*), suggesting this fermentation broth contained selectively active compounds (Thompson et al., 1997). The taxonomic classification of this isolate identified it as an actinomycete, *Saccharopolyspora spinosa*, one of the few members of an obscure genus (Mertz and Yao, 1990). After extensive purification and structure resolving techniques, the insecticidal components of the broth were identified as glycosylated macrocyclic lactones, commonly referred to as spinosyns (Figure 2, (Kirst et al., 1991)). The wild type strain of *S. spinosad* produces 15 different types of spinosyns that vary by methylation sites on various parts of the molecule (Salgado and Sparks, 2005). Spinosad is a mixture of spinosyns A and D (85:15 respectively), which are the most biologically active and most abundant of the naturally occurring spinosyns (Thompson et al., 1997, Salgado and Sparks, 2005). Mutant strains were identified in a strain improvement program that lacked *O*-methyltransferase activity. These mutants produced 10 additional spinosyns. Sinefungin is a compound that specifically inhibits 4-*O*-methyltransferase activity (Chen 1989). Four novel

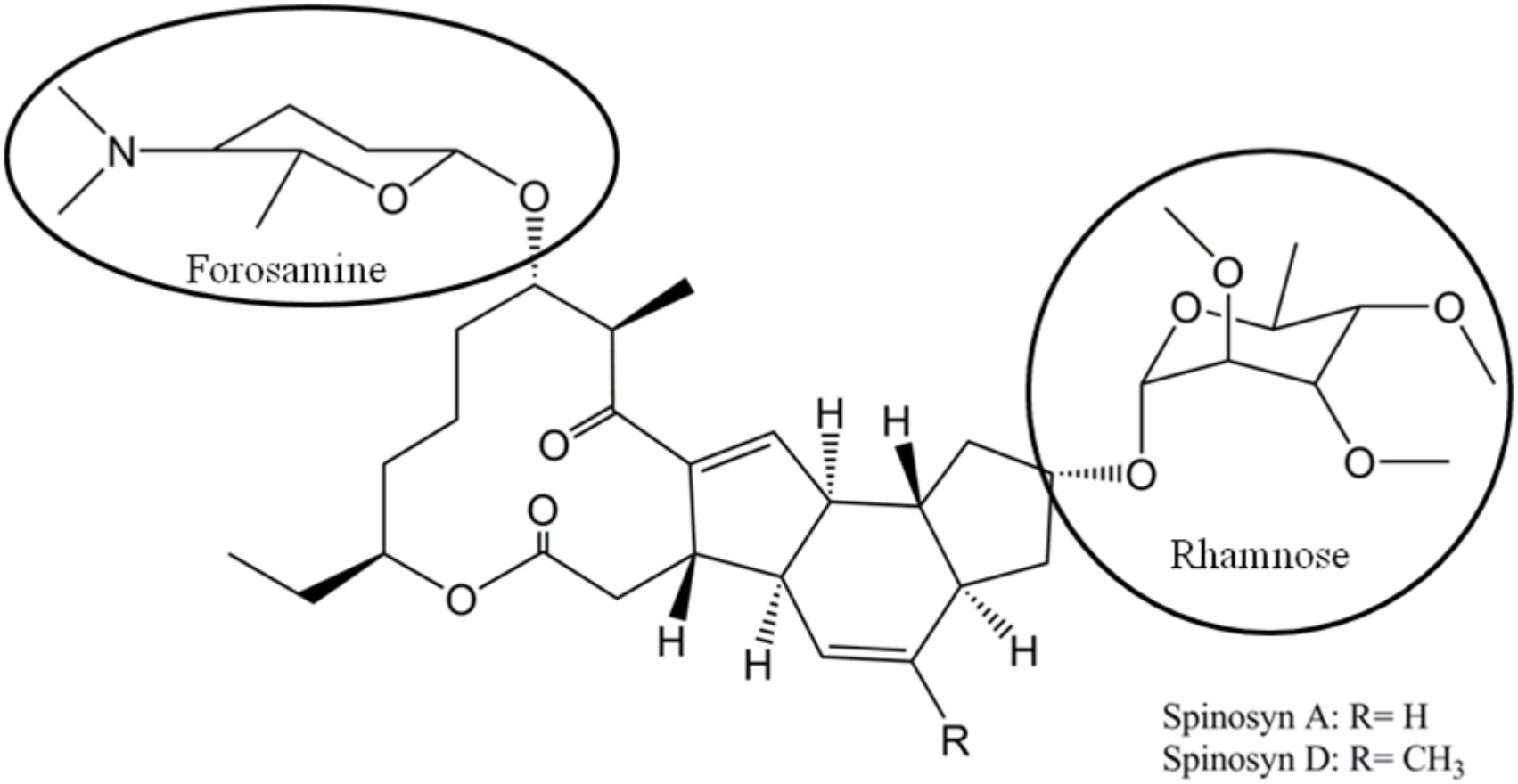

Figure 1.2 Chemical structure of the components of spinosad, spinosyns A and D. The forosamine and rhamnose sugars are circled, and the remaining structure is the tetracycle backbone.

spinosyns were produced by the addition of sinefungin to growth medium. A closely related species, *Saccharopolyspora pogona*, produces over 30 different types of 21-butenyl spinosyns, suggesting other species in the *Saccharopolyspora* genus may produce a wide variety of valuable spinosyns (Lewer, 2003).

1.3.1.2 Biosynthesis and Derivation

The biosynthetic pathway of spinosad has been elucidated to further understand how this unique structure forms within the organism and genetic engineering of various *Saccharopolyspora* species has generated a wide variety of inconceivable spinosyns that show greater biological activity than spinosyn A. The 3′-*O*-ethyl and the 2′-3′-4′-tri-*O*-ethyl analogs of spinosyn A are an order of magnitude more potent than spinosyn A (Salgado and Sparks, 2005). The details of these studies have been thoroughly reviewed (Crouse et al., 2001, Huang et al., 2009, Salgado and Sparks, 2005).

1.3.1.3 Structure Activity Relationships

Modification of spinosyns focuses on the three major structural areas of the molecule: the tetracycle backbone, the forosamine and rhamnose sugars (Figure 1.2). Testing more than 1000 unique derivatives have allowed for investigating structure activity relationships. Relative toxicity in the following sections is based on tests with larvae of the tobacco budworm, *Heliothis virescens*. Reduction, epoxidation, or hydroxylation of the 5,6 double bond on the tetracycle doesn't increase activity. The length of the alkyl group at C21 is positively correlated with toxicity. No other modification of the tetracycle significantly increases toxicity (Salgado and Sparks, 2005).

The forosamine sugar is important to insecticidal activity. Removal of this group makes the compound completely ineffective (Sparks et al., 1999). Replacing the sugar with groups such as acetate or various *N*-alkyl chains greatly reduce the effectiveness of these compounds (Kirst et al., 2002). Substitution of the *N*, *N*-dimethyl group on the forosamine sugar with a hydroxyl group significantly reduces activity. Thus, the native configuration of this moiety seems to be optimal.

The rhamnose sugar appears to be more amenable to modifications that increase toxicity. Much like the forosamine sugar, complete removal of the rhamnose sugar renders the compound ineffective. Additionally, substituting rhamnose with other sugars also abolishes effectiveness. Desmethoxy analogues at the 2′ and 3′ positions of spinosyns H Q and J are equally or marginally more toxic than spinosyn A. However, demethylation of 2′, 3′, and 4′ reduce their effectiveness (Sparks et al., 1999). The most important position appears to be the 3′ position. The potency of these compounds increases by switching from methoxy to ethoxy or propoxy at the 3′ position. Longer and branching chains reduced toxicity while bond reduction (i.e. alkylation) or halogenation had muted effects (Salgado 2005). Further evidence of the importance of the 3′ position is evidenced in the similar toxicity profiles of 2′, 3′, 4′-tri-*O*-ethyl and 3′-*O*-ethyl substitutions. Addition of a hydroxyl group at the 6′ position creates a compound significantly more toxic than spinosyn A (Anzeveno and Green, 2002).

1.3.1.4 Mode of Action

1.3.1.4.1 Symptoms

Spinosyns have an excitatory effect on insect behavior. Exposure to spinosad in American cockroaches (*Periplaneta americana*), fruit flies (*Drosophila melanogaster*), and

tobacco budworm (*Heliothis virescens*) produced similar symptoms in all three insects. The progression of overt symptoms include: an elevated body posture due to leg extension, uncoordinated walking, prostration, followed by fine tremors and paralysis. Other symptoms were excessive and uncoordinated wing beating, abdominal inflation, and diuresis (Salgado, 1998). These symptoms correlate well with electrophysiological measurements in American cockroaches. An electromyogram shows the tarsal flexor muscles controlling the legs are excessively stimulated by spinosyn A. The metathoracic crural neurons controlling these muscles also show increased excitability. The muscle showed no activity when ipsilateral nerve 5 was cut, suggesting this activity was controlled by a circuit consisting of the thoracic ganglion, ipsilateral nerve 5 and the tarsal flexor muscle (Salgado, 1998).

1.3.1.4.2 Interactions at the nAChR

The neural circuitry that gives rise to symptoms of spinosyn poisoning is due to an influx of sodium in the post-synaptic neuron to cause depolarization of neurons. Sodium influx due to spinosyns are blocked by the nAChR antagonist α-bungarotoxin providing evidence for the nAChR as the target site (Salgado and Saar, 2004). The level of excitability at the nAChR relative to spinosyn A is positively correlated with insecticidal activity in *H. virescens* (Salgado and Sparks, 2005). Spinosyns bind to nAChRs, but ACh is still able to bind, indicating spinosyns interact with the nAChR at a different location than ACh or imidacloprid (Salgado, 1997). Spinosad most likely interacts with different populations of nAChRs with different subunit composition (Salgado and Saar, 2004). It appears that spinosad works at a unique target site that is not shared with any other insecticide (Orr et al., 2009).

A strain of *D. melanogaster* possessed a chromosomal inversion that disrupted open reading of *Dα6* that would code for a protein that is truncated after TM2. Flies that were homozygous for this deficient gene were more than 1180-fold resistant to spinosad, but still susceptible to neonicotinoids (Perry et al., 2007). Spinosad sensitivity was selected for after *D. melanogaster* were mutagenized with EMS and spinosad resistance was due to the nAcRα-30D^{DAS1} allele, which is a null mutation of the *Dα6* subunit. Flies homozygous for the nAcRα-30D^{DAS1} allele were more than 370-fold less sensitive to spinosad and the ventral ganglion were insensitive to spinosad (Watson et al., 2010). These results suggest that nAChRs with *Dα6* are the unique target site of spinosad.

1.3.1.4.3 Interactions at the GABA-Receptor

A secondary site of action for spinosyns appears to be GABA-gated chloride channels, although this mechanism is less understood. GABA commonly works by reducing neuroexcitability by counteracting the influx of positive ions by allowing negatively charged chloride ions to enter the cell. Spinosyn A diminishes GABA-induced current in a time and dose-dependent manner in small neurons in the cockroach, *P. americana* (Watson and Salgado, 2001). Spinosyns may act by reducing the inhibitory effect of GABA, thus synergizing the effect of nAChR activation. However, the precise mechanism is poorly understood (Watson and Salgado, 2001).

1.3.1.5 Utility of Spinosyns

Since their initial discovery, the efficacy of spinosyns has been highly lauded for its effectiveness against many pest species in many agricultural commodities. It is effective against

major pests such as tobacco budworm (*Heliothis virescens*), fall armyworm (*Spodoptera frugiperda*) and diamond back moth (*Plutella xylostella*) (Salgado and Sparks, 2005). Spinosyns are relatively safe to many agriculturally beneficial insects such as the lady beetle (*Coccinella septempunctata*), insidious flower bug (*Orius insidiosus*), and crysopid (*Chrysoperla rufilabris*) (Williams et al., 2003, Thompson et al., 2000). This selective toxicity coupled with low impact on non-target vertebrates makes spinosyns an ideal insecticide (Salgado and Sparks, 2005). They are labeled for use in more than 30 countries on more than 250 crops such as corn, cotton, tobacco, cruciferous vegetables and many citrus varieties. It can also be applied to turf, stored grains, fire ant mounds and other locations around the home and garden (Bret et al., 1997, DowAgroscience, 2001, DowAgroscience, 2004). There is no cross resistance to spinosad in strains resistant to pyrethroids, abamectin, cyclodienes, or organophosphates (Scott, 1998).

1.3.2 Nicotine, Nicotinoids, and Neonicotinoids

1.3.2.1 Introduction

Tobacco (*Nicotiana tabacum*) was introduced to Europe from North America, reportedly by Sir Walter Raleigh. The insecticidal properties of aqueous extracts of tobacco leaves were first documented around 1690. The active compound was isolated in 1828 and named nicotine for Jean Nicot who introduced tobacco to France. Nicotine was synthesized in a laboratory in 1904. Dried tobacco leaves may contain up to 6% nicotine by weight (Ujvary, 1999). Nicotine and some related alkaloids are used as plant defenses against insects that damage leaf tissue through a jasmonic acid cascade pathway (Baldwin et al., 1997).

Nicotinoids refer to compounds that share structural similarity to nicotine (Figure 1.3). This group includes anabasine, nornicotine, dihydronictyrine, and others. Only nicotinoids with a basic pyridyl-nitrogen that is protonated at physiological pH are insecticidal (Yamamoto, 1999). The first synthetic nicotine derivative to have insecticidal action was nithiazine. The structure is a simple nitromethylene enamine. Nithiazine has systemic activity in plants and was significantly more toxic than parathion to houseflies (*Musca domestica*), corn earworm (*Helicoverpa zea*), and aphids (*Aphis pisum*) (Jeschke and Nauen, 2005). However, nithiazine is not photostable and only of limited use (Kollenmeyer et al., 1999). The promising potency and wide spectrum of efficacy of nithiazine prompted further development. More than 2000 compounds were screened for activity in bioassays with green rice leafhopper (*Nephotettix cincticeps*). The compound that had the most optimal balance of potency, environmental stability, systemic activity and other parameters was given the name imidacloprid (Figure 1.3 (Kagabu, 1999)). Imidacloprid is a prototypical member of compounds called neonicotinoids (Tomizawa and Yamamoto, 1993). Neonicotinoids are the fastest growing class of insecticides with >17% share of the insecticide market and >$1B in annual revenue. There are currently seven commercially sold neonicotinoids: imidacloprid, thiacloprid, thiamethoxam, nitenpyram, acetamiprid, clothianidin, and dinotefuran (Figure 1.3, (Jeschke and Nauen, 2008).

1.3.2.2 Classification

Neonicotinoids can be classified based on the structure of the heterocyclic N-substituent or the pharmacophore (Figure 1.4). The ring structure can vary as well, but is not used in classifying neonicotinoids. Heterocyclic N-substituent groups include chloronicontyl (6-chloro-pyrid-3-ylmethyl; e.g. imidacloprid), thianicotinyl (2-chloro-1,3-thiazol-5-ylmethyl;

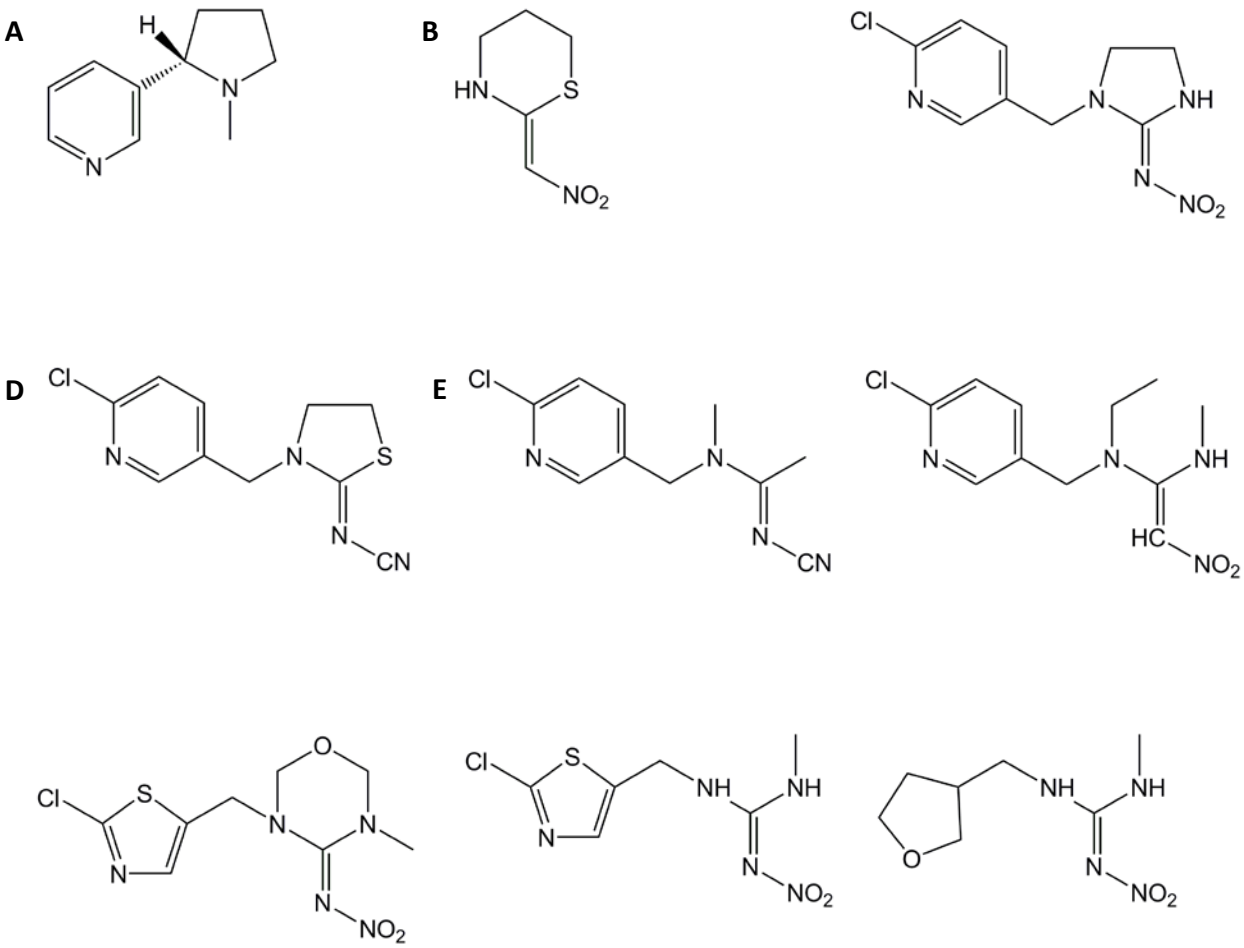

Figure 1.3 Chemical structures of A) nicotine and eight neonicotinoids: B) nithiazine, C) imidacloprid, D) thiacloprid, E) acetamiprid, F) nitenpyram, G) thiamethoxam, H) clothianidin, I) dinotefuran.

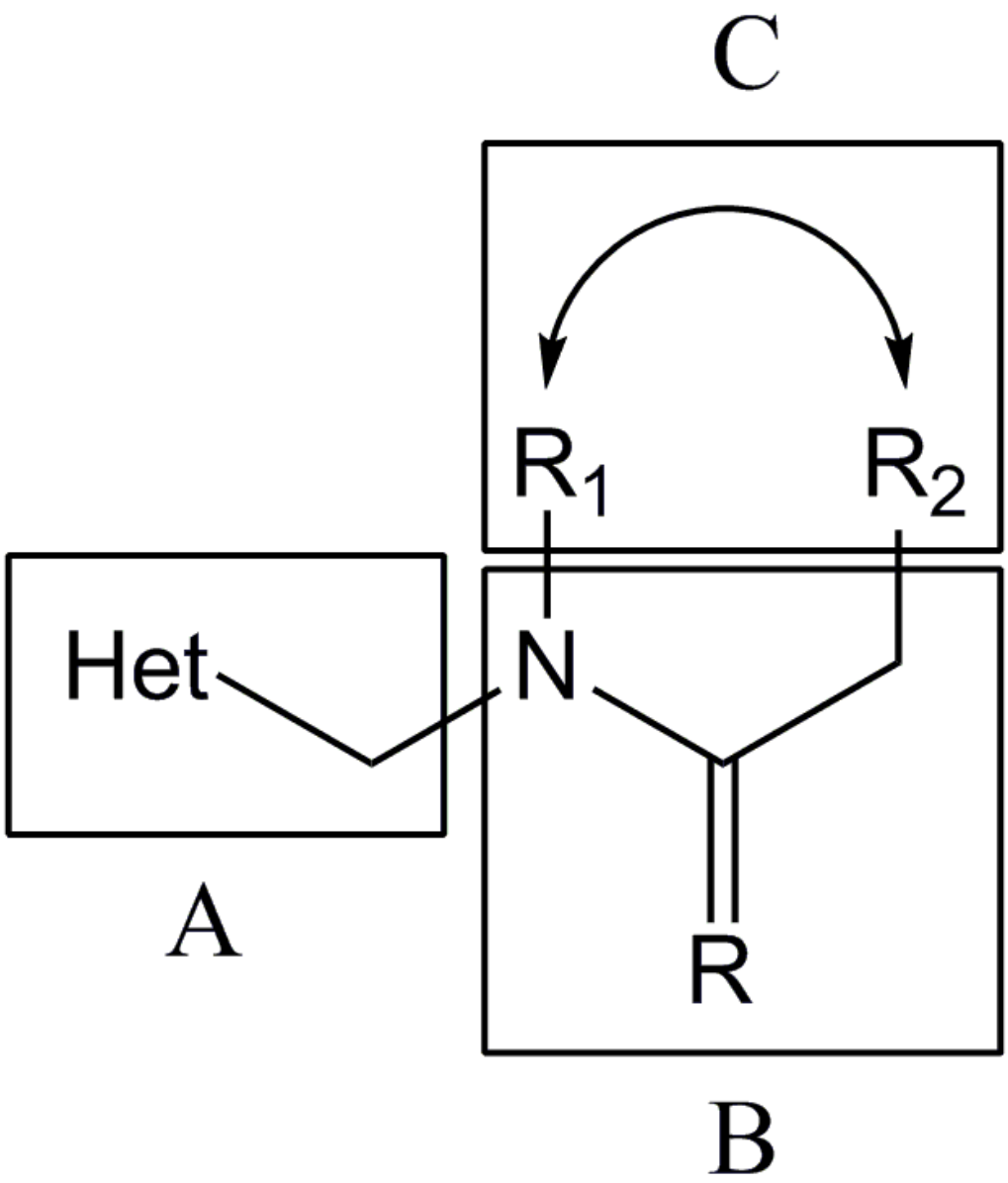

Figure 1.4. Structural components of neonicotinoids used for classification. A) Heterocyclic N-substituent groups include chloronicontyl (Figure 3C-F), thianicotinyl (Figure 3G, H) and furanicotyl (Figure 3I). B) Pharmacophores groups include nitroguanidines or nitroimines (Fig 3C, G-I), nitromethylenes (Figure 3F), and cyanoamidines (Figure 3D, E) C) The ring system is not used for classifying neonicotinoids, but is important for toxicity (Kagabu et al., 2002, Kagabu, 2003).

thiamethoxam) and furanicotyl ((+)-tetrahydro-fur-3-ylmethyl; dinotefuran). This system of neonicotinoids classification is largely based on market introduction date rather than physiological differences due to different groups. The heterocyclic group stabilizes the interaction of neonicotinoids with the receptor by forming an H-bond with a tyrosine residue at the receptor binding site (Kagabu et al., 2002). Pharmacophores groups include nitroguanidines or nitroimines ($C=NNO_2$; imidacloprid), nitromethylenes ($C=CHNO_2$; nitenpyram), and cyanoamidines (C=NCN; acetamiprid). The pharmacophore is an electron withdrawing group that influences insect toxicity (nitromethylene > nitroguanidine > cyanoamidine), photostability and environmental fate (Kagabu, 1999, Shiokawa et al., 1994, Tomizawa and Yamamoto, 1993). It has been suggested that the pharmacophore definition is more informative because physiological and environmental interactions are classified more accurately (Nauen et al., 2001).

1.3.2.3 Structure-Activity Relationships

Structure activity relationships have been evaluated using thousands of compounds that vary on each of the three regions of the neonicotinoids (Figure 1.4). The type of heterocyclic group is important for H-bond formation and stable receptor interactions. Nitromethylene compounds with Cl in the *para* position of the 6-chloro-3-pyridyl group is 10x more potent for receptor binding and stimulation as well as up to 3 orders of magnitude more toxic than compounds without Cl (Buckingham et al., 1995, Tomizawa and Yamamoto, 1993). Switching the Cl with a methyl group lowers binding affinity but toxicity remains comparable to compounds with Cl (Tomizawa and Yamamoto, 1993). Switching the position of the nitrogen in the pyrid-3-ylmethyl from the *meta* position to *ortho* or *para* position or removal all together lowers the binding affinity to the receptor by 2, 3 and 2 orders of magnitude, respectively.

Toxicity of the *ortho*-nitrogen or nitrogen free compounds is significantly reduced. Adding Cl to nitrogen free compounds does not restore binding affinity or toxicity (Tomizawa and Yamamoto, 1993). It has been confirmed that the position of this nitrogen is required for hydrogen bonding analogous to the carbonyl oxygen of ACh (Tomizawa et al., 1999).

The strength of nAChR binding depends on the type of pharmacophore on the neonicotinoid. The negatively charged nitro or cyano pharmacophore interacts with a positively charged amino acid (lysine, arginine or histidine) at the insect nAChR binding site (Tomizawa et al., 2000). Interestingly, in mammals the nAChR binding site is slightly negatively charged due to tryptophan at the binding site so the strength of interactions with negative pharmacophores is reduced. It is this charge difference between insect and mammalian nAChRs that most likely accounts for the selectivity of neonicotinoids to insects and low toxicity to mammals (Tomizawa and Casida, 2005, Tomizawa and Casida, 2003). Changing the pharmacophore has a dramatic impact on receptor binding and insect toxicity. Although the binding affinities for nitromethyl, nitroimine, and cyanoamindine can vary, they are all highly toxic to insects. Compounds with the replacement of this group to an amide or cyanomethyl groups are practically nontoxic to insects (Tomizawa and Yamamoto, 1993).

The number and types of atoms in the ring structure influences toxicity (Figure 4C). Replacement of the sulfur from nithiazine with carbon abolishes α-bungarotoxin displacement and insect mortality (Buckingham et al., 1995). In nitromethylene analogs of imidacloprid, the type of atom (N, S, C, O) in the imidazolidine ring at the 3 position does not alter toxicity, but binding affinity can range over an order of magnitude (Tomizawa and Yamamoto, 1993). Modification of the region between the nitrogens at the 1 and 3 positions of the imidazolidine ring of the nitromethylene derivatives of imidacloprid can influence insect toxicity and receptor

binding 100 and 1000-fold respectively. A six membered diazacyclohexyl ring is more toxic than a 5 or 7 membered ring. Adding an isopropyl or *n*-butyl group to the 5 position of diazacyclohexyl ring does not alter toxicity, but binding is greatly reduced. Interestingly, substituting any number of other alkyl groups from a methyl through phenyl moiety or alcohols dramatically lowers toxicity and binding. Adding dimethyl groups to the 4 or 5 position of the imidazolidine ring modestly reduces toxicity but significantly reduces binding. The extra groups create more hydrophobic compounds, but there is no correlation between hydrophobicity and toxicity or affinity. These results indicate the bulkiness of the ring creates a "sterically restricted" region that interferes with receptor binding and toxicity (Kagabu et al., 2002). The same rules of steric restriction apply to non-cyclic neonicotinoids such as acetamiprid and thiamethoxam. These compounds form "quasi-cyclic" conformations when bound to the receptor (Kagabu, 2003).

1.3.2.4 Mode of Action

Extensive biochemical and electrophysiological data indicate neonicotinoids act as agonists of the nAChR. Imidacloprid at 200 nM was effective at displacing the nAChR antagonist, α-bungarotoxin (αBGT), in cockroach neurons and from housefly and honey bee head membrane preparations (Bai et al., 1991, Tomizawa and I., 1992, Tomizawa and Yamamoto, 1993). The displacement of αBGT was seen in many orders of insects from locust (*Locusta migratoria*) to tobacco hornworm (*Manduca sexta*) (reviewed in (Tomizawa and Casida, 2003). Displacement of αBGT suggests that imidacloprid interacts strongly with the ligand-binding domain of the nAChR. In housefly head membrane preparations, rapid receptor binding of imidacloprid has a high specificity and affinity. The IC_{50} for imidacloprid is more

than 2 orders of magnitude lower than other compounds that interact at the ligand-binding site (Liu and Casida, 1993). The strength of the interaction between the neonicotinoids and its receptor is important for insect toxicity. The IC_{50} for 20 nitromethylene and nitroguanidine derivatives based on the structure of imidacloprid was recorded and compared to the 1-hour knockdown (KD_{50}) for that compound. Compounds with low IC_{50} values had correspondingly low KD_{50}values, suggesting these two parameters are related. There was no correlation between IC_{50} and 24-hour mortality (LD_{50}) (Liu et al., 1993). In retrospect, it seems obvious that neonicotinoids would interact with the nAChR. The distance between the nitrogens of the pharmacophore and the heterocyclic group are within the range of the distances between the ammonium nitrogen and carbonyl oxygen of ACh (Tomizawa et al., 2000).

Neonicotinoids interact with different types of nAChRs. Imidacloprid depolarizes cultured cockroach (*Periplaneta americana*) neurons that contain both αBGT sensitive and insensitive receptor subtypes (Buckingham et al., 1997). Interestingly, imidacloprid does not compete for binding sites on αBGT sensitive nAChRs, which suggests it has a unique binding site (Salgado and Saar, 2004). Distinct imidacloprid binding sites on αBGT sensitive nAChRs were also reported from *D. melanogaster* brains (Zhang et al., 2004). High and low affinity binding sites were found in membrane preparations from the green peach aphid (*Myzus persicae*). These studies indicate that neonicotinoids interact differently with receptors composed of various subunits. This is an especially interesting concept considering the number of nAChR subunit genes in insects and the ability to generate multiple subunit types from a single gene through alternative splicing and A-to-I RNA-editing.

Photoaffinity labeling can help identify specific subunits involved in neonicotinoid binding. Affinity chromatography using nicotinoid-agarose columns purified a receptor from *D.*

melanogaster head membranes with 3 peptides that had molecular weights of 61, 66, and 60 kDa. This purified fraction was preincubated with or without competitive cholinergic ligands. Proteins were then photolabeled with [^{125}I]azidonicotinoid (AzNN). The 66 kDa protein was specifically labeled with [^{125}I]AzNN. This protein corresponds to the size of the protein predicted from the deduced protein sequence of the Dα2 subunit (Tomizawa and Casida, 1997). [^{3}H]AzNN was used to label receptor subunits in *Myzus persicae*, *Homaldisca coagulata*, *Musca domestica*, and *D. melanogaster*. For each species, [^{3}H]AzNN was bound to a single, highly specific binding site, suggesting a conserved binding site is shared between these insects. The size of the peptide photolabeled by [^{3}H]AzNN in *M. persicae*, *H. coagulata*, *M. domestica*, and *D. melanogaster* was 45, 56, 66, and 66 kDa, respectively. It is unclear which subunit in *M. persicae* (45 kDa) or *H. coagulata* (56 kDa) is labeled due to ambiguities of estimating molecular weights from SDS-PAGE and the smallest nAChR subunit weight from the deduced protein sequence from *M. persicae* is 56 kDa (Huang et al., 2000). The identification of the same size protein labeled in *D. melanogaster* is consistent with the previous report that the Dα2 subunit is the site of neonicotinoid binding (Tomizawa and Casida, 2001).

The specific residues involved in neonicotinoid binding have been investigated with site-directed mutagenesis of acetylcholine binding protein (AChBP) from the sea slug, *Aplysia californica*. The crystal structure of AChBP is well understood and AChBP is model protein to understand ligand interactions in the extracellular domain of nAChRs (Hansen et al., 2005, Brejc et al., 2001). Residues in loop E of AChBP were mutated to methionine except methionine at position 116 was mutated to leucine (M116L). A Y195F mutation was also included in these experiments. The relative photolabeling was investigated using single mutants and combinations of mutants. The Y195F and M116L/Y195F mutants had very low levels of photolabeling while

M116L by itself increased photolabeling. In all cases, the inclusion of the Y195F mutant significantly reduced photolabeling to <15% relative to the WT. These results indicate that Y195 of AChBP is a critical residue for initiating and stabilizing interactions with the ligand and other nearby residues (Tomizawa et al., 2009).

Crystal structures of AChBP of *A. californica* and the snail, *Lymnea stagnalis*, complexed with imidacloprid have been used to study the mechanism of neonicotinoid interaction with specific residues in the ligand binding domain of nAChRs. The 2-nitroimino-imidazolidine group stacks with the conserved tyrosines at positions 185 and 188 in *L. stagnalis* and *A. californica*, respectively. Hydrogen bonds are formed between tryptophan in loop B and the nitrogen of the pyridine ring of imidacloprid. The basis of selectivity and increased affinity to insect nAChRs is imparted by the interaction of the partial negative charge of the nitro group of imidacloprid with basic residues in loop B of insect nAChR subunits that correspond to Q55 and Q57 of 188 in *L. stagnalis* and *A. californica*, respectively (Matsuda et al., 2009).

The importance of the basic residue in loop D to insect selectivity is supported by electrophysiology of mutated vertebrate subunits. Site directed mutagenesis was used to create a Q79R mutants of the chicken α7 subunit. The Q79 residue of chicken α7 subunit aligns with Q55 of *L. stagnalis*, as previously discussed. The mutant and WT chicken α7 subunit were expressed in *Xenopus* oocytes and studied with two-electrode voltage clamp. The Q79R mutant had significantly increased sensitivity (pEC_{50}) to nitenpyram and channel conductance (I_{max}) due to imidacloprid or nitenpyram binding than the WT channel, suggesting these compounds are potent agonists of the mutated nAChR (Shimomura et al., 2002).

The role of loop D is important for imidacloprid sensitivity in β subunits. Wild type nAChRs composed of chicken α4β2 subunits are sensitive to ACh but not imidacloprid. When

both T77K/Rand E79R mutants were introduced to the β2 subunits, the receptors became sensitive to imidacloprid. A similar increase in sensitivity was observed in *D. melanogaster* Dα2/chicken β2 receptors. Imidacloprid sensitivity was increased in Dα2β2 receptors with T77N or R, T77N, E79R and T77R, E79V mutations in the chicken β2 subunits. These mutations imitate the basic residues of insect nAChRs (Shimomura et al., 2006).

Residues in loop C can enhance interactions with imidacloprid. Introduction of E219P in loop C into chicken α4 subunits increases sensitivity and conductance due to imidacloprid. The E219S or T mutations did not change receptor properties. However, when expressed with combinations of T77R/N/K and E79V/R, the E219S/P/T mutations in the β subunit produced conducting receptors and became sensitive to imidacloprid. The conductance of α4 (E219P)/β2 (T77N; E79R) receptors treated with imidacloprid was twice as much as conductance due to ACh. These results suggest complex and synergistic interaction of imidacloprid with many regions of the ligand-binding domain (Toshima et al., 2009). These findings are supported by analyzing reverse mutations in Dα2. Introducing a P242E mutation into Dα2/chicken β2 receptors significantly reduces imidacloprid sensitivity and channel conductance. It is hypothesized that the P242E repulses other acidic residues on loop F or the partial negative charge on the pharmacophore of neonicotinoids (Shimomura et al., 2004).

1.3.2.5 Utility of Neonicotioids

The properties of neonicotinoids lend themselves to a diverse number of convenient and effective application methods. Neonicotinoids can be applied in with a number of methods and is sold as granules, wettable powders, water soluble pouches, liquids, and capsules under the brand names such as Admire®, Capstar®, Mospilan®, and Calypso®. Neonicotinoids are used on

more than 140 crops in 120 countries. Neonicotinoids are useful in controlling pests of cotton, corn, wheat, rice, and most major crops. Neonicotinoids are widely used in home settings. Control of domestic pests such as termites, ants, cockroaches, clothes moths, and lawn grubs is easily obtained with neonicotinoids (Agrawal and Tilak, 2006). The lack of skin irritation and selectivity for insect over mammalian nAChRs makes neonicotinoids ideal compounds for effective and lasting control of blood feeding arthropods of veterinary importance such as fleas, lice and ticks (Hopkins et al., 1996, Jacobs et al., 1997, Hanssen et al., 1999). Upon patent expiration, generic versions of neonicotinoids are expected to decrease costs and increase use (Elbert et al., 2008).

1.4 Changes in nAChRs Associated with Insecticide Resistance

1.4.1 Importance and Challenges of nAChRs in Insecticide Toxicity

Most cases of insecticide resistance are to compounds that work at acetylcholinesterases (organophosphates and carbamates), sodium channels (DDT and pyrethroids), and GABA-gated chloride-channels (cyclodienes and phenylpyrazoles) (Whalon et al., 2008). Acetylcholinesterases are monomeric proteins and sodium channels are dimmers. GABA-gated chloride-channels in insects may be heteropentamers with combinations of 3-4 unique subunits. This is an interesting point because there are single target sites for acetylcholinesterases and two potential sites on sodium channels, but there more than 243-1024 potential combinations of GABA-gated chloride-channel subunits. What makes nAChRs interesting as a target site is that more than 100-248,000 combinations are theoretically possible from the range 10-12 nAChR subunits found in insect genomes. This presents an especially unique and difficult challenge to elucidate which subunit(s), subunit combination(s), and subunit stoichiometry(s) are involved in

insecticide resistance. Currently, not much is known about the native composition of insect nAChRs (Sattelle et al., 2005).

1.4.2 Spinosad Resistance

There are many reports of resistance to spinosad in field collected animals including thrips (Bielza et al., 2007), diamondback moth (Zhao et al., 2002), and cotton bollworm (Wang et al., 2009). In the remainder of this section, I will focus on studies where efforts have been made to understand the mechanism of spinosad resistance. Laboratory strains of diamondback moth (Shono and Scott, 2003, Zhao et al., 2002), tobacco budworm (Wyss et al., 2003) and houseflies (Shono and Scott, 2003) have been selected for resistance to spinosad. In all three organisms, resistance was inherited as a monogenic recessive trait. In diamondback moth and houseflies, inhibitors of metabolic detoxification enzymes were used to investigate if metabolism was responsible for resistance (Shono and Scott, 2003, Zhao et al., 2002). The results indicate P450s, esterases and GSTs are not involved in resistance, thus, spinosad resistance is likely due to a target site resistance mechanism. Also, resistance to spinosad does not confer significant cross resistance to other compounds which further indicates a unique target site and resistance mechanism (Shono and Scott, 2003, Zhao et al., 2006).

The nAChR subunits in house fly do not appear to be involved with resistance. Spinosad resistance in the *rspin* strain was due to a recessive factor on autosome 1. There was no cross resistance to insecticides with known target sites in the *rspin* strain. P450s and esterases are not involved in spinosad resistance in the *rspin* strain, suggesting target site modification is the underlying cause of spinosad resistance (Shono and Scott, 2003). The *rspin* strain showed no differences compared to a spinosad susceptible strain in gene sequence, alternative splicing,

RNA-editing, or expression levels of *Mdα2, Mdα5, Mdα6 and Mdβ3* that would be consistent with the pattern of inheritance for spinosad resistance (Gao et al., 2007a, Gao et al., 2007b, Gao et al., 2007c). GABA-gated chloride channels do not appear to be involved with spinosad resistance in house flies as *Rdl* is found on autosome 4 (Gao et al., 2007d). As such, the cause of spinosad resistance in house flies remains unclear (Scott, 2008).

Western flower thrips collected from lettuce crops in New South Wales, Australia, over the 2002-03 season showed low levels of spinosad resistance (<2-fold), but resistance rose to 87-fold by 2005-06. These thrips were subject to spinosad selection in the lab. This selection increased the Resistance Factor (RF) from 1.8 in the field collected strain to 62.4 after spinosad selection (Herron and James, 2005). These results were especially discouraging because spinosad that is the product most compatible with IPM practices for western flower thirps control (Herron and James, 2007).

Spinosad overuse (>10 applications/year) has resulted in high levels of spinosad resistance in western flower thrips in greenhouses in Spain. These field collected thrips were subject to spinosad selection for only 4 generations of selection and resulted in the R1S strain with more than 153,000-fold spinosad resistance at the highest level of spinosad that was able to be applied (Bielza et al., 2007). Resistance in R1S is not able to be overcome with metabolic synergists, suggesting that target site insensitivity may be the underlying cause of spinosad resistance in this population of western flower thirps (Bielza et al., 2005). Spinosad resistance is autosomal, monofactorial, nearly completely recessive (Bielza et al., 2007). The resistance mechanism appears to carry very little, if any, pleiotropic effects. Female thrips of the spinosad resistant R1S strain laid significantly more eggs and significantly more of those eggs hatched to first instar larvae when compared to the spinosad susceptible MLFOM and the acrinathrin

resistant ACRI strains (Bielza et al., 2008b). These results should be taken cautiously as they are genetically unrelated strains. Five populations of thrips were created with varying proportions of resistant individuals (0, 25, 50, 75 and 100%) and bioassayed every other generation for 8 generations. Resistance factors remained relatively constant in each population throughout the duration of this experiment (Bielza et al., 2008a).

Target site resistance was also implicated in a spinosad resistant strain of western flower thrips in Japan. Spinosad toxicity did not change when thrips from the ICS strain were treated with PBO, DEM or DEF which are inhibitors of cytochrome P450 monooxygenases, glutathione *S*-transferases, and esterases, respectively (Zhang et al., 2008a). However, the ICS strain only had a resistance ratio of 14, so vast differences in bioassays with synergists may not be easily discerned. The pattern of inheritance of spinosad resistance in ICS is autosomal and incompletely dominant (Zhang et al., 2008a).

The tobacco budworm (*Heliothis virescens*) does not show wide spread spinosad resistance in the field. A subpopulation of tobacco budworms that were collected from tobacco fields in North Carolina in 1996 and 97 were selected with spinosad every generation for 13 generations. The spinosad selected strain was 669-fold resistant to spinosad compared to the original field collected strain. Spinosad resistance was autosomal, monofactorial and partly recessive. The resistance mechanism was stable when spinosad selection pressure was removed for 5 generations, suggesting little fitness cost associated with the resistance mechanism (Wyss et al., 2003).

Many populations of diamondback moth in California, Florida, Texas and Mexico were susceptible to spinosad (Zhao et al., 2006, Eziah et al., 2008). However, diamondback moths collected from Camilla, GA in 2001, Irwin, GA in 2002, Mitchell, GA in 2003, and Oxnard, CA

in 2004 showed high levels of spinosad resistance (8000 to 24,000-fold resistance (Zhao et al., 2006)). Diamondback moths across Hawaii showed moderate to high levels of spinosad resistance (12 to 6,000-fold). A population of diamondback moth collected from Pearl City, HI in 2001 were >1,000-fold resistant to spinosad. The Pearl City strain was selected with spinosad at 100 and 200 ppm in successive generations to create the Pearl-Sel strain with more than 18,000-fold resistance to spinosad. Reciprocal backcrosses showed inheritance of spinosad resistance was autosomal, incompletely recessive and monofactorial. Resistance levels were unchanged by the synergists PBO and DEF, suggesting enhanced metabolism is not a major resistance mechanism in the Pearl-Sel strain (Zhao et al. 2002).

The CH_1 strain of diamondback moth was collected from Cameron Highlands, Malaysia in November 2002. The CH_1 strain is more than 20,000-fold resistant to spinosad. Reciprocal backcrosses showed inheritance of spinosad resistance was autosomal and monofactorial. Resistance is incompletely dominant at low doses but completely recessive at high doses (Sayyed et al., 2004).

The Multan strain (MN) of diamondback moth was collected from cabbage crops in Pakistan in October 2006. The MN strain was selected for 10 generations with spinosad. This selection regiment resulted in the Spino-SELF strain with more than 1,900-fold resistance to spinosad. Reciprocal backcrosses showed inheritance of spinosad resistance was autosomal, incompletely recessive and monofactorial. Interestingly, PBO and DEF were able to significantly increase the toxicity of spinosad to the Spino-SEL strain suggesting that enhanced metabolism is a major resistance mechanism in the Spino-SEL strain. When spinosad selection pressure was relaxed for 8 generations, spinosad resistance levels declined from 1983-fold to 8-fold implies a substantial fitness cost for the spinosad resistance mechanism in the Spino-SEL strain. The

measured fitness costs in the Spino-SEL strain included low pupal weight, and low proportion of egg hatching (Sayyed et al., 2008).

1.4.3 Neonicotinoid Resistance

Despite being increasingly used to control major agricultural and horticultural pests since the introduction of imidacloprid in 1991, neonicotinoid resistance remains geographically restricted to a few pests in the field. For example, tobacco whiteflies (*Bemisia tabaci*) collected from Spain in 1995 showed moderate levels of imidacloprid resistance (Cahill et al., 1996). High levels of resistance (>100-fold) to neonicotinoids were seen a few years later from the same region (Elbert and Nauen, 2000, Nauen et al., 2002). A strain collected from Israel was >1000-fold resistant to a few neonicotinoids (Rauch and Nauen, 2003). Fortunately, high levels of resistance are not wide spread and susceptibility is commonly restored (Li et al., 2000, Li et al., 2001). Whiteflies from California to Arizona showed no neonicotinoid resistance despite regular use of those compounds (Palumbo et al., 2001). However, the potential to select high levels of resistance in the lab suggests there is the potential to develop high levels of resistance in field populations (Jeschke and Nauen, 2005, Nauen and Denholm, 2005, Jeschke, 2007).

Aphids are ideal species to develop resistance to neonicotinoids. The systemic activity of this class targets these plant fluid feeding insects. Aphids have high rates of reproduction and they can be transported on plants undetected over large geographic ranges. The peach-potato aphid, *Myzus persicae*, can have low levels of neonicotinoid tolerance (<10-fold) that did not correlate with specific biochemical markers (Nauen et al., 1998). Tolerance is different than resistance. Tolerance is the ability of a strain to survive a higher dose of an insecticide than a susceptible strain that has no biochemical or genetic basis. Tolerance values are usually 2 to 10-

fold. A highly sensitive susceptible strain can amplify the perception of tolerance. The tolerance in *M. persicae* is likely due to feeding on tobacco and not field exposure to neonicotinoids (Nauen et al., 1998). Interestingly, high levels of resistance have not been found in any other aphid species (Wang et al., 2002, Weichel and Nauen, 2003, Nauen et al., 2003).

Insects utilize two major mechanisms for neonicotinoid resistance: enhanced metabolism and reduced target site sensitivity. The importance of these factors varies for each pest, and examples will be presented below

Detoxification by cytochrome P450s is an important neonicotinoid resistance mechanism. For example, high levels of imidacloprid resistance (>1000-fold) in multiple strains of whiteflies (*B. tabaci*) was largely suppressible with PBO (Nauen et al., 2002) and resistant strains had increased 5-hydroxy imidacloprid production. These results suggest detoxification via P450s is a major resistance mechanism in the whitefly (Rauch and Nauen, 2003). Overexpression of *CYP6CM1* was highly correlated with resistance levels, and resistance was specifically attributable to the r-Q allele that was only seen in the resistant strains (Karunker et al., 2008). CYP6CM1 heterologously expressed in *E. coli* metabolized imidacloprid. Therefore, overexpression of the imidacloprid detoxifying CYP6CM1 is likely responsible for imidacloprid resistance in *B. tabaci* (Karunker et al., 2009).

Extremely high levels of imidacloprid resistance were seen in the FRC clone of the green peach aphid, *Myzus persicae*. The FRC strain only showed 28% mortality at the extremely high dose of imidacloprid (i.e. 1000 mg/L), so the actual Resistance Ratio was not able to be calculated. Imidacloprid had a lower affinity to whole body membrane preps of the FRC strain due to the loss of the loss of the high affinity binding site. Cloning of the *Mpβ1* subunit revealed an R81T mutation in ligand binding loop D. Interestingly, the threonine at this position has been

implicated as an important residue responsible for the reduced affinity of imidacloprid to vertebrate receptors (Tomizawa and Casida, 2005, Tomizawa and Casida, 2003). Thus, the R81T makes these receptors more "vertebrate-like". Additionally, the production of 4/5-hydroxyimidacloprid was significantly higher in the FRC strain. Expression of CYP6CY3 was 28-fold overexpressed due to an 8-fold increase in gene amplification. Therefore, the high levels of resistance in the FRC strain are due to target site insensitivity (R81T of *Mpβ1*) and increased metabolic detoxification via CYP6CY3 (Bass et al., 2011).

A field collected strain of the brown planthopper, *Nilaparvata lugens*, was selected with imidacloprid for 25 generations that resulted in a 72-fold increase in LD_{50}. The resistance is most likely due to a target site mutation(s) because resistance was unable to be overcome with synergists that inhibit P450s, esterases, and glutathione-*S*-transferases (Liu et al., 2003). The resistant strain was selected with imidacloprid for another ten generations to create the R-T35 strain, which was 250-fold resistant. In whole body membrane preps of the susceptible strain (S), two high affinity and saturable binding sites for [^{3}H]imidacloprid were identified. However, there was no detectible specific binding of [^{3}H]imidacloprid in membrane preps from the R-T35 strain. Membrane preps from the R-T35 strain were able to bind [^{3}H]epibatidine, which suggests the membranes are functional and contain nAChRs. This binding was significantly lower than from the S strain. Degenerate primer amplification followed by 5′/3′ RACE was used to clone the cDNAs of 5 nAChR subunits (α1-4 and β1) from the S and R-T35 strains. A single Y151S mutation in Nlα1 and Nlα3 of the R-T35 strain was associated with resistance. The R-T35 strain was homozygous for the Y151S mutation of Nlα1. The wild type Nlα1 and the mutant Nlα1^{Y151S} subunits were expressed in *Xenopus* oocytes with rat β2. The Nlα1^{Y151S} expressing oocytes had 100-fold and 32-fold lower specific binding of [^{3}H]imidacloprid and [^{3}H]epibatidine,

respectively. It is postulated that the reduction in binding affinity for both compounds is due to the disruption of interaction with the critical Y151 residue in the binding site of the nAChR as inferred from comparison to nAChRBP from *Lymnea stagnalis* (Liu et al., 2005).

The Nlα1^{Y151S} mutation reduces the potency of neonicotinoids in electrophysiological studies. The maximum inward current (I_{max}) was measured in *Xenopus* oocytes expressing wild type Nlα1 or the Nlα1^{Y151S} mutation along with rat β2. There were no differences in I_{max} due to ACh application between both receptor subtypes but there was a small but significant increase in EC_{50}. Oocytes expressing Nlα1^{Y151S} containing receptors had an 8-fold reduction in I_{max} and 2.7-fold increase in the EC_{50} due to imidacloprid application, thus confirming the previously reported reduction in binding affinity (Liu et al., 2005) correlated with reduced receptor sensitivity. The same relative reduction in I_{max} and increase in EC_{50} was seen for all 7 commercially available neonicotinoids besides dinotefuran (Liu et al., 2006).

The Y151S mutation was also seen in Nlα3. Early attempts at oocyte expression with Nlα3 were unsuccessful. Expression of Nlα8 alone creates minimally functional receptors. However, receptor function is greatly enhanced when Nlα3 is expressed in oocytes along with Nlα8 and rat β2. Unlike Nlα1^{Y151S}, the Nlα3^{Y151S} mutation had a 2-fold higher EC_{50} for ACh. However, much like Nlα1^{Y151S}, the Nlα3^{Y151S} mutation had a 7-fold reduction in I_{max} and 10-fold increase in the EC_{50} for imidacloprid. The I_{max} was reduced and the EC_{50} was increased dramatically and significantly for all seven commercially available neonicotinoids. Therefore it seems reasonable to conclude that the Y151S mutation creates neonicotinoids insensitive channels regardless of which subunit it is contained (Zhang et al., 2009). However, Nlα8 used in this study has a tyrosine at a similar position to Y151 of Nlα1 and its role in receptor function was not investigated.

Not all changes to Y151 reduce imidacloprid sensitivity. The major difference between tyrosine and serine is the presence of a phenyl group between the amino acid backbone and hydroxyl group. The effect of the phenyl group on receptor function was investigated using oocytes expressing an Nlα1^{Y151F} mutation. These receptors had a reduced I_{max} and increased EC_{50} to ACh but no dramatic changes in those parameters in response to imidacloprid. It is speculated that the polar portions of tyrosine and serine are important properties of those side chains that mediate interaction of ACh with the receptor via water molecules (Song et al., 2009).

The Y151 site of Nlα1 aligns to a methionine residue in acr18 α-subunit from *Caenorhabditis elegans*. Site directed mutagenesis was used to generate a Y151M mutation into Nlα1. The Nlα1^{Y151M} containing receptors were able to respond to ACh and bind imidacloprid with high affinity, no inward current was detected when imidacloprid was applied to oocytes expressing Nlα1^{Y151M}. These results indicate that imidacloprid acts as an antagonist to receptors with Nlα1^{Y151M} which is the first time a mutation changes the mode of action of an insecticide. The authors suggest that the Nlα1^{Y151M} mutation may not lead to a resistant phenotype because it also has a significant reduction in ACh-invoked current (Zhang et al., 2008b).

Although these studies have shown that the Y151S mutant of Nlα1 and Nlα3 can produce resistant strain, these mutations have not been found in field collected strains of brown planthopper. The imidacloprid resistant strains CHN-2 and IND-11 did not have the Y151S mutation and specific binding of imidacloprid was similar to a susceptible strain. These two resistant strain had 5-7 higher rates of 7-ethoxycoumarin *O*-deethylation, thus suggesting imidacloprid resistance in these populations may be due to P450-mediated metabolism (Puinean et al., 2010).

Chapter 2

Research Goals

2.1 General Research Goals

There is overwhelming genetic, biochemical, and physiological evidence that indicates the nAChR is the target site of both spinosad and imidacloprid. The goal of my project was to determine the subunit composition of these target sites. Elucidating the composition of nAChRs involved in the target site of these insecticides is a substantial challenge because they are pentameric complexes that can be composed from a pool of 10-16 subunits, which may undergo an array of post-transcriptional modifications. Moreover, these receptors may be distributed in specific tissues.

2.2 Rationale

The target site for spinosad in *Drosophila melanogaster* has been identified as the α6 subunit. However, this research was performed on laboratory created strains (Perry et al., 2007, Watson et al., 2010). While *D. melanogaster* is usually a very reliable model organism, there have been cases where the results achieved in *D. melanogaster* are not transferable to other insects. For example, the *kdr* mutation in the voltage-sensitive sodium channel is found as an L1014F mutation in many pests that have evolved resistance to pyrethroids (Soderlund and Knipple, 2003). None of the mutations in sodium channel of *D. melanogaster* that are responsible for pyrethroid resistance are seen in any other pest, thus demonstrating some of the potential limitations of using *D. melanogaster* as a general model of insecticide resistance (Pittendrigh et al., 1997). Therefore, I will investigate whether deletion of *α6* is also the mechanism of spinosad resistance in a pest insect, the diamondback moth.

Mutagenesis and laboratory selection studies have identified subunits involved in sensitivity to spinosad and imidacloprid in *D. melanogaster* (Perry et al., 2008, Perry et al., 2007). These studies have relied on selection for the insensitive phenotype followed by the identification of the mutant gene underlying the change in sensitivity. I propose to systematically study the contribution of each subunit by utilizing RNAi against each receptor. Should this method prove useful, it will allow me to intimately and precisely define the target sites of spinosyns and neonicotinoids.

Transcripts of nAChRs undergo extensive and highly conserved post-transcriptional modifications such as alternative splicing and A-to-I RNA editing. While this phenomenon has been observed in many species, the role of these modified transcripts is mostly unexplored. To date, the only role that has been identified for post-transcriptional modification of nAChR transcripts is that nAChRs containing an N133D substitution via A-to-I RNA editing are less sensitive to imidacloprid than wild-type receptors (Yao et al., 2009). However, it is unknown whether or not RNA editing of nAChRs causes a change in spinosad or imidacloprid sensitivity *in vivo*.

2.3 Specific Goals

2.3.1 Sequencing *Pxyla6* from Diamondback Moth

The Pearl-Sel strain of diamondback moth, *Plutella xylostella*, is more than 18,000 fold resistant to spinosad. The pattern of inheritance for spinosad resistance in the Pearl-Sel strain of diamondback moth has been described as autosomal, monofactorial, completely recessive, and unable to be overcome with metabolic inhibitors, suggesting that modification of the target site is responsible for high levels of spinosad resistance (Zhao et al., 2002). Previous work has shown

that deletion of the *Dα6* subunit in *D. melanogaster* causes high levels of spinosad resistance (Perry et al., 2007). Therefore, I will sequence the *Pxyla6* subunit from two susceptible stains and a spinosad resistant strain of diamondback moth to determine if any mutations in this gene are responsible for spinosad resistance. If there are any differences between the resistant and susceptible strains that are consistent with resistance, I will use the F_1 backcross to the resistant strain paired with a diagnostic spinosad bioassay to determine if these differences are genetically linked with spinosad resistance.

2.3.2 Cloning of nAChRs from *Tribolium castaneum*

I will evaluate the contribution of nAChRs other than the α6 subunit on spinosad toxicity by using RNAi in the red flour beetle, *Tribolium castaneum*. I chose the red flour beetle because RNAi is robust in this species, while RNAi is largely unexplored and undocumented in diamondback moth. The first step I will take to this end, will be to clone all 12 nAChRs from the red flour beetle. These clones will be used as templates for the production of dsRNA that will be injected into pupae to silence the expression of these subunits. I will start my investigation by performing RNAi against *Tcasα6* because the deletions of α6 subunit are involved in spinosad resistance (Perry et al., 2007). Additionally, I will explore the possibility of novel transcript variants across all 12 nAChR subunits.

2.3.3 RNAi of *α6* in *T. castaneum* and *D. melanogaster*

As previously stated, the *Dα6* subunit is involved in spinosad resistance. Therefore, I will use RNAi against *α6* to evaluate the utility of RNAi for assessing the role of each subunit in spinosad toxicity in *T. castaneum* and *D. melanogaster*. If the level of spinosad resistance in *α6*

deficient beetles and flies is similar to *Dα6* nulls, I will systematically study the contribution of each additional subunit in spinosad resistance. I will employ RNAi by injection of dsRNA into *T. castaneum* pupae and the Gal4-UAS system in *D. melanogaster*. Additionally, I will drive the expression of dsRNA for *Dα6* in all tissues and only in the nervous system and then expose these flies to spinosad to determine if there is any change in spinosad resistance due to tissue-specific reduction in *Dα6* expression.

2.3.4 RNAi of *dAdar* in *D. melanogaster*

The *Dα6* nAChR subunit contributes to spinosad sensitivity and undergoes A-to-I RNA editing at seven sites in the ligand-binding domain. I will use RNAi of *dAdar* to evaluate if A-to-I RNA editing of this target affects spinosad sensitivity in *D. melanogaster*. I will use the Gal4-UAS system to reduce expression of *dAdar* in the entire insect, the nervous system, cholinergic neurons, muscle, and glia to evaluate if there are any tissue specific effects on spinosad sensitivity. I will also test imidacloprid sensitivity on these *dAdar* deficient flies.

2.4 Significance

If mutations of *Pxyla6* are genetically associated with spinosad resistance, this will be the first description of a spinosad resistance in a field selected resistant pest. If, there are no mutations of *Pxyla6* that are congruent with a description of resistance, this will add to the surprising uncertainty of identifying the mechanism of spinosad resistance because house flies have high levels of spinosad resistance, but there are no mutations in *Mdα6* associated with resistance (Shono and Scott, 2003, Scott, 2008).

The utility of RNAi has yielded tremendous insight to the function of many genes in many organisms. If I am able to utilize RNAi against the *α6* subunit in *T. castaneum* and *D. melanogaster* in a manner consistent with previous research, I will be able to systematically study the contribution of nAChR subunits to insecticide toxicity either individually or in any imaginable combination. This would provide tremendous insight into dissecting the function of extremely complex numerical diversity of nAChRs.

There has been little work on the effect of A-to-I RNA editing on insecticide sensitivity. By using RNAi to eliminate this process that modifies nAChR subunits, I will be able to further explore the role of another source of receptor diversity may have on receptor function. These results will add to the rapidly growing field of research on the effect that A-to-I RNA editing has on insect behavior and physiology in a novel and important niche that is largely unexplored.

Chapter 3

Transcripts of the nicotinic acetylcholine receptor subunit gene *Pxylα6* with premature stop codons are associated with spinosad resistance in diamondback moth, *Plutella xylostella*[1]

3.1 Introduction

Nicotinic acetylcholine receptors (nAChRs) form cationic-selective, ligand-gated ion channels that are involved in cholinergic transmission in the insect central nervous system. nAChRs can be homopentamers of α subunits (Fayyazuddin et al., 2006) or heteropentamers of both α and β subunits (Thany et al., 2006). Sequencing insect genomes has revealed 10-12 subunit genes in insects (Sattelle et al., 2005, Jones et al., 2006, Shao et al., 2007). Insect nAChR subunit genes utilize alternative exons, A-to-I RNA-editing, and other post-transcriptional modifications to generate a diverse pool of transcripts from the 10 to 12 subunit genes in each of their genomes (Sattelle et al., 2005, Rinkevich and Scott, 2009). Transcripts with premature stop codons, which either have some unknown function or are targets for non-sense mediated decay (Chang et al., 2007), have been noted in *D. melanogaster* (Grauso et al., 2002), *A. mellifera* (Jones et al., 2006), and *Tribolium castaneum* (Rinkevich and Scott, 2009).

Macrocyclic lactones with 12-carbon macrocycles that act on nAChRs, such as spinosad, are a modern and rapidly expanding class of insecticides (Crouse et al., 2001, Salgado and Sparks, 2005). They are highly regarded for their effectiveness against insect pests, minimal environmental impact and low vertebrate toxicity (Salgado and Sparks, 2005, Casida and Quistad, 2004). Although macrocyclic lactone insecticides exert their toxic effects through interactions with nAChRs (Salgado, 1997), they bind to receptors with unique subunit

[1]Previously published as Rinkevich, F. D., et al. (2010) Invert. Neurosci. 10**,** 25-33.

composition (Salgado and Saar, 2004) or at locations on the receptor outside of agonist binding domains (Orr et al., 2009). A study on *Drosophila melanogaster* has found that loss of *Dα6* via a premature stop codon results in a strain resistant to spinosad (>1000-fold) (Perry et al., 2007).

Diamondback moth (*Plutella xylostella*) is a very important pest of cruciferous crops such as cabbage, cauliflower and broccoli (Talekar and Shelton, 1993). It can be one of the most difficult pests to sustainably control with insecticides due to the rapid rate at which it evolves resistance. Field populations of diamondback moth from across the globe have become resistant to organophosphates (Sayyed et al., 2005), pyrethroids (Kwon et al., 2004), *Bacillus thuringiensis* Cry1Ac and Cry1Ba toxins (Wang and Wu, 2007), and neonicotinoids (Ninsin et al., 2000). Diamondback moth infested 48% of the canola crop in Australia in 2002, and control measures cost up to $28 AUD per acre (Gu et al., 2007). Management of diamondback moth across the world exceeds $1 billion annually (Talekar and Shelton, 1993). The Pearl-Sel strain of diamondback moth was collected from Pearl City, Hawaii in October 2000. Following spinosad selection, the Pearl-Sel strain was >13,000-fold resistant to spinosad. Resistance was found to be completely recessive, autosomal, monofactorial and could not be overcome with metabolic inhibitors piperonyl butoxide (PBO) and S,S,S-tributyl phosphorotrithionate (DEF), suggesting resistance was due to an altered target site (Zhao et al., 2002). A similar pattern of inheritance was seen in the Field CH1 strain collected from the Cameron Highlands of Malaysia with > 20,000 fold resistance to spinosad (Sayyed et al., 2004).

Spinosad resistance has been documented in the tobacco budworm (*Heliothis virescens*) (Wyss et al., 2003) and house fly (*Musca domestica*) (Shono and Scott, 2003). In both studies, the patterns of inheritance of spinosad resistance were similar to cases of spinosad resistance in the Pearl-Sel and CH1 strains of *P. xylostella*.

Herein, I describe the full length cDNA of the *Pxylα6* subunit from the insecticide susceptible Geneva 88 and Wapio strains and the spinosad resistant Pearl-Sel strain of diamondback moth. I describe the differences in cDNA sequences between diamondback moth strains that may act as potential resistance mechanisms. An F_2 backcross and bioassay are performed to demonstrate genetic association of transcript differences with spinosad resistance.

3.2 Materials and Methods

3.2.1 Insects

Three strains of diamondback moth (*P. xylostella*) were used in this experiment. The Geneva 88 (G88) strain is insecticide susceptible (Zhao et al., 2002). The Pearl-Sel strain is >18,000-fold resistant to spinosad (Zhao et al., 2002). The Wapio strain is resistant to indoxacarb and permethrin, but not spinosad (Chen, unpublished). Colonies were reared on an artificial diet and environmental conditions as previously described (Shelton et al., 1991). Five individual fourth instar larvae were collected in a 1.5 ml centrifuge tube with 200 μl of absolute ethanol and stored at -80 ^{o}C. Prior to collection, the Pearl-Sel strain was treated with a discriminating dose (10 ppm) of spinosad (Zhao et al., 2002) and survivors were collected and stored in ethanol at -80 ^{o}C. Larvae were rehydrated by replacing the ethanol with decreasing concentrations of ethanol (95%, 70%, 35%, and 0%) every 2 minutes before RNA extraction.

3.2.2 Reverse transcription, PCR and cloning

Total RNA was extracted from five larvae using 1 ml of TRIzol as described by the manufacturer (Invitrogen, Carlsbad CA). cDNA was synthesized with 5 μg of total RNA using

SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad CA) according to the manufacturer's directions, except the reverse transcription step was extended to 3 hrs.

Degenerate primers were designed based on conserved amino acid residues in the α6 subunits from an alignment of Agamα6 (AY705401), Amelα6 (NM_001080095), Bmα6 (NM_001098372), Dα6 (NM_164874), and Tcasα6 (EF526088). MaF2 and MaR1 were also used (Gao et al., 2007c). The PxylActinF/R primer pair amplified a 500 bp fragment of actin cDNA (AB282645) that was used as a positive control in every PCR. Primer sequences are shown in Table 3.1. PCR was performed using the following iCycler thermocycler program (Bio-Rad, Hercules CA): initial denaturation 95^{o}C for 2 min; 35 cycles of 95^{o}C for 30 s, 46^{o}C for 30 s, and 72^{o}C for 90 s; final extension of 72^{o}C for 10 min. PCR products were separated on a 1% agarose gel stain stained with ethidium bromide (10 μg/ml) and visualized under UV light. The PCR reaction resulting from the primer pair Pxylα6ProF8/MaR1 was purified using Wizard PCR Purification kit (Promega, Madison WI). Purified PCR products were cloned into pGEM-T (Promega, Madison WI) according to the manufacturer's directions with an overnight ligation at 4^{o}C. JM109 competent cells were transformed with 2 μl of the ligation reaction according to the manufacturer's directions. Individual colonies were spotted to a fresh plate then screened for positive inserts using the colony directly in a 15 μl PCR reaction with GoTaq (Promega, Madison WI) and T7 and SP6 primers (Table 3.1). Thermocycler conditions were as follows: initial denaturation 95^{o}C for 2 min; 35 cycles of 95^{o}C for 30 s, 42^{o}C for 30 s, and 72^{o}C for 60 s; final extension of 72^{o}C for 10 min. PCR products were visualized on an agarose gel as described above. Colonies with the correct insert were grown in 3 ml of Luria-Beranti medium supplemented with ampicillin at a final concentration of 50 μg/ml. Plasmids were purified using PureYield Miniprep kit (Promega, Madison WI). Plasmids were sequenced in both directions

Table 3.1 Sequences of primers used.

Primer name	**Sequence (5′ – 3′)**
PxylA6ProF1	SNGARTAYGGNGGNGT
PxylA6ProF2	YGAYGAYCARCAYTGYGA
PxylA6ProF3	GAARTTYGGNWSNTGGACNTAYGAYGGNAA
PxylA6ProF4	HCAYHARATGCCNCCNTGGATHAARW
PxylA6ProF5	RTTYGGNYTNACNYTNCARCARATHATHGA
PxylA6ProF7	CAYGARATGCCNCCNTGGATHAARWS
PxylA6ProF8	GAAGTTCGGATGCTGGACTTACGATGGAA
PxylA6ProF9	CCVAACAARCRYTGGAARCCIGA
PxylA6ProF10	GACCARCAYTGYGAIATGAARTT
PxylA6ProF11	TTCAAYTGYATYATGTTCATGGT
PxylA6ProF12	GAAGTTCGGATGCTGGACTTACGATGG
PxylA6ProR1	CANGGNGGCATYTCR
PxylA6ProR3	ANGGNGGCATYTCRTGDATRTCNGCN
PxylA6ProR4	CNGCYTCYTCRTCNGCYTTYTTCATNCKN
PxylA6ProR5	NGTRTTYTTYTTNCCNGGCATNCCDATN
PxylA6ProR8	TTCCATCGTAAGTCCAGCATCCGAACTTC
MaF2	ATGAARTTYGGNWSNTGGACNTAYGA
MaR1	GCNACCATRAACATDATRCARTTRAA
PxylA6A	CGAGAACGGGGGAGACCTGTCTGAC
PxylA6AR	GTCAGACAGGTCTCCCCCGTTCTCG
PxylA6B	CGATCATGATCCGGCGCCGGACGCT
PxylA6BR	AGCGTCCGGCGCCGGATCATGATCG
PxylA65UTRF	AAAAGTCCCGCGCCGAGCGC
PxylA65UTRF2	CCGTCGGGGCCGGTCA
PxylA63UTRR	TTTTTTTTTTTACATTTTAATAATAATTACATGAATGGGCGTGAT
PxylA63UTRR2	GCGTGATCTAAGATTAGTTCTCTCGTGAG
PxylActinF	AGCCGCCCTCGTAGTGGACAATGG
PxylActinR	GATGGCGTGGGGAAGAGCGTAACC
T7	TAATACGACTCACTATAGGG
SP6	TATTTAGGTGACACTATAG

using T7 and SP6 primers at Cornell University's Biotechnology Resource Center. Sequences were aligned using MegAlign (Lasergene, Madison WI).

Gene specific primers Pxyla6A, Pxyla6AR, Pxyla6B and Pxyla6BR were designed based on the sequence obtained from the above protocol (Table 3.1). 5' and 3' RACE was performed using FirstChoice RLM-RACE (Ambion, Austin TX). 5' RACE was performed using Pxyla6BR and the kit provided 5' RACE-outer primer in the first round of PCR (initial denaturation 95^{o}C for 2 min; 35 cycles of 95^{o}C for 30 s, 58^{o}C for 30 s, and 72^{o}C for 3.5 min; final extension of 72^{o}C for 10 min) in 100 μl PCR reactions using GoTaq. Five μl of the first round product was used in a second round 100 μl PCR reaction using Pxyla6AR and the kit provided 5' RACE-Inner primer using the same thermocycler conditions as above. PCR products were visualized, purified, cloned, and sequenced as described above.

3' RACE reverse transcription was performed using FirstChoice RLM-RACE according to the manufacturer's directions. Two ul of the reverse transcription product were used in a 50 μl first round PCR reaction with the Pxyla6A primer and the kit provided 3' RACE-outer primer. Thermocycler conditions were as follows: initial denaturation 95^{o}C for 2 min; 35 cycles of 95^{o}C for 30 s, 58^{o}C for 30 s, and 72^{o}C for 1.5 min; final extension of 72^{o}C for 10 min. A second round reaction was performed using 5 μl of the first round reaction and the Pxyla6B and the kit provided 3' RACE-inner primers. Thermocycler conditions were as described above. PCR products were visualized, purified, cloned, and sequenced as described above.

Alignments of the 5' and 3' RACE products yielded the tentative full length cDNA. Primers were designed based on the ends of the tentative full length cDNA (PxylA65UTRF, PxylA65UTRF2, PxylA63UTRR, and PxylA63UTRR2). First round PCR was performed in 50 μl GoTaq reactions using two μl of cDNA with the primer combination of PxylA65UTRF and

PxylA63UTRR and the following thermocycler conditions: initial denaturation 95^{o}C for 2 min; 35 cycles of 95^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 2 min; final extension of 72^{o}C for 10 min. Second round PCR was performed in 100 µl GoTaq reactions using five ul of first round PCR product with the primer combination of PxylA65UTRF2 and PxylA63UTRR2 and the following thermocycler conditions: initial denaturation 95^{o}C for 2 min; 35 cycles of 95^{o}C for 30 s, 47^{o}C for 30 s, and 72^{o}C for 2 min; final extension of 72^{o}C for 10 min. PCR products were visualized, purified, cloned, and sequenced as described above. Cloning of full length cDNAs was performed on at least two independent batches of five larvae for each strain. At least five clones from each batch were sequenced for each strain. Sequences were aligned using MegAlign to identify alternative exon use, putative A-to-I RNA-editing, alleles and other transcript modifications. Putative phosphorylation and *N*-linked glycosylation sites were identified using NetPhos 2.0 (Blom et al., 1999) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively.

3.2.3 F_1 backcross and bioassay

The spinosad resistance status of each strain was evaluated before crosses were initialized (Zhao et al., 2006). Pearl-Sel males were mass crossed to G88 females. Female F_1s were backcrossed to Pearl-Sel males. The resulting offspring were treated with a diagnostic dose of spinosad as previously described (Zhao et al., 2006). A small subset (c.a. 100) of animals at each generation and treatment were collected and stored in ethanol at -80^{o}C. RNA extraction, reverse transcription, PCR, cloning and sequencing were performed as described above.

3.3 Results

3.3.1 *Pxylα6* from spinosad susceptible strains

The consensus full length cDNA and features of the *Pxylα6* deduced amino acid sequence from the G88 and Wapio strains are shown in Figure 3.1 (GenBank Accession GQ247883). The 5' and 3' UTRs are 114 and 97 bp, respectively. There was no variation in the length or sequence of both the 5' and 3' UTRs between strains. The amino acid sequence of the protein (deduced from the nucleotide sequence of *Pxylα6*, Isoform I) shares highest identity to *Dα6* and *Bmoα6* (82.4% and 95.4%, respectively Table 3.2) and groups with α6 orthologs from other species in an alignment of insect nAChRs (Figure 3.2). There are potentially eight serine, six threonine, and three tyrosine phosphorylation sites, and 3 *N*-linked glycosylation sites on the protein. The length of the open reading frame is variable due a splice site variation discussed below. Neither cassette exons, retained introns nor premature stop codons were observed in the *Pxylα6* transcripts in the G88 and Wapio strains, although these were observed in subunit transcripts from *A. mellifera* (Jones et al., 2006), *D. melanogaster* (Grauso et al., 2002), *M. domestica* (Gao et al., 2007c) and *T. castaneum* (Rinkevich and Scott, 2009).

Alternative exons were only observed for exon 3 (3a and 3b). Only isoforms I and III were observed (Figure 3.3A and C; (Rinkevich and Scott, 2009)). A summary of the percent identity of the nucleotide and amino acid sequence of *Pxylα6* and the frequency of exon use in each strain is shown in Table 3.3. The nucleotide sequence of *Pxylα6* exon 3a is 66.7% and 88.9% identical compared to *Dα6* exon 3a and *Bmoα6* exon 3a, respectively. The translated amino acids coded by *Pxylα6* exon 3a are and 93% and 100% identical to both *Dα6* exon 3a and *Bmoα6* exon 3a, respectively. The frequency of exon 3a usage was 0.09 in G88 and 1.0 in

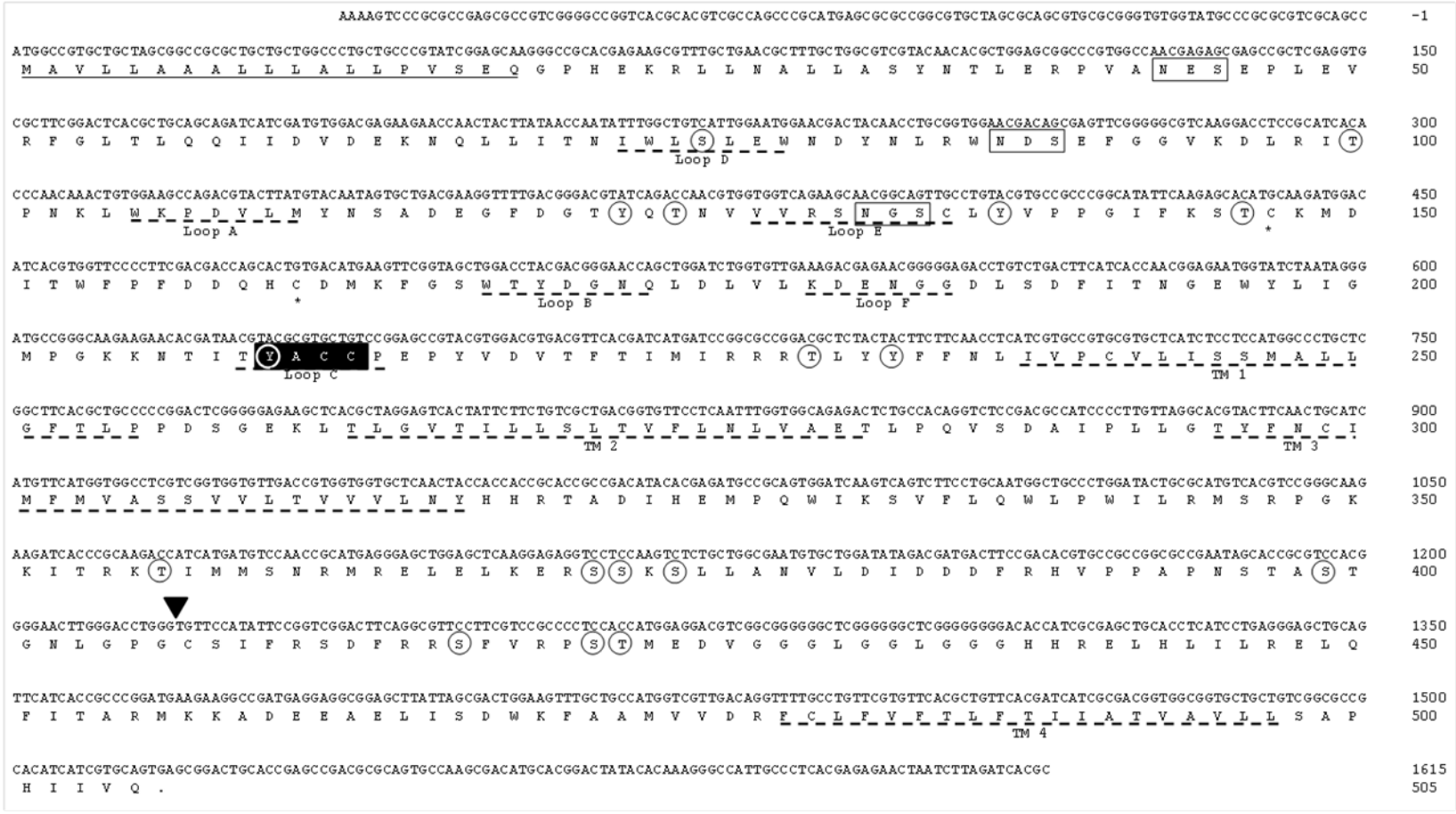

Figure 3.1 Nucleotide and deduced amino acid sequence of *Pxyla6* (GenBank Accession GQ228552). Numbers on the right indicate the nucleotide (upper) and amino acid (lower) position. The N-terminal signal peptide is underlined with a solid line. Putative *N*-glycosylation and phosphorylation sites are boxed and circled, respectively. Ligand binding loops and transmembrane domains are underlined with dashed lines. The YxCC motif is shaded and the vicinal Cys residues are designated with asterisks. The location of the intron 10 splice site variation that adds 10 amino acids to the protein is indicated by an inverted triangle.

Table 3.2 Comparison of the percent identity of the deduced amino acid sequence of *Pxyla6* to *D. melanogaster* and *B.mori* nAChR subunits. The numbers to the upper right portion of the diagonal line of black boxes indicate the % identity to *B.mori* nAChR subunits. The numbers to the lower left portion of the diagonal line of black boxes indicate the % identity to *D. melanogaster* nAChR subunits.

	Pxylα6	Bmα1	Bmα2	Bmα3	Bmα4	Bmα5	Bmα6	Bmα7	Bmα8	Bmα9	Bmβ1	Bmβ2	Bmβ3	
Pxylα6		36.4	35.6	36.6	33.6	39.8	95.4	67.4	35.0	17.7	35.8	17.9	13.4	Pxylα6
Dα1	36.8		54.4	56.9	53.3	38.9	36.9	37.2	56.4	19.1	40.1	16.6	14.6	Bmα1
Dα2	36.6	52.4		52.0	47.5	34.7	35.7	36.6	50.8	18.4	39.5	16.6	13.1	Bmα2
Dα3	37.2	54.9	47.6		69.3	38.0	36.9	39.2	57.3	19.6	41.6	15.3	14.1	Bmα3
Dα4	36.2	52.9	44.9	62.9		36.5	36.7	37.0	53.6	18.7	41.5	16.8	14.1	Bmα4
Dα5	65.2	34.0	34.2	24.7	33.1		38.6	40.1	36.8	18.5	35.6	16.4	13.1	Bmα5
Dα6	82.4	38.1	34.8	36.4	37.4	67.6		66.4	35.7	17.9	35.9	17.1	12.6	Bmα6
Dα7	66.4	35.6	37.1	37.3	36.2	74.4	66.6		36.2	17.5	35.0	14.0	13.1	Bmα7
Dβ1	36.0	41.2	39.7	43.3	41.6	34.1	35.4	33.7		19.1	39.3	15.3	14.1	Bmα8
Dβ2	35.0	54.8	51.7	56.3	54.0	31.5	36.2	35.2	39.0		18.4	24.9	21.9	Bmα9
Dβ3	18.1	17.6	19.7	15.6	18.8	16.5	17.6	15.8	17.4	19.9		16.1	14.9	Bmβ1
													23.6	Bmβ2
	Pxylα6	Dα1	Dα2	Dα3	Dα4	Dα5	Dα6	Dα7	Dβ1	Dβ2	Dβ3			Bmβ3

Figure 3.2 Phylogeny based on deduced amino acid sequence of the all nAChRs from *B. mori, T. castaneum, D. melanogaster*, and *A. mellifera*. The α6 subunit from *P. xylostella* (*Pxylα6*) is located in the Dα5-7 Group near its *B. mori* ortholog (*Bmα6*).

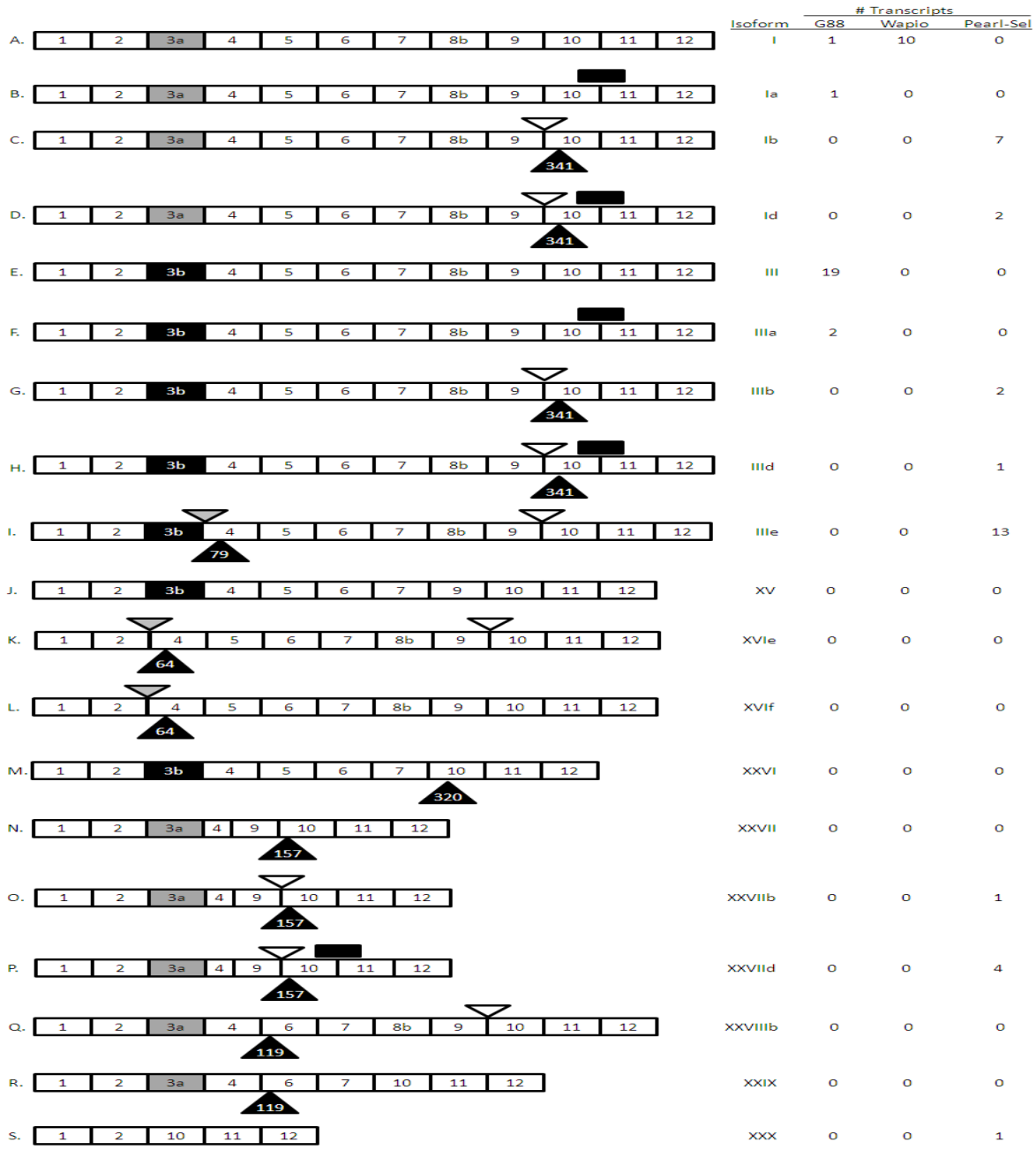

Figure 3.3 Schematic diagrams of *Pxyl*α*6* transcripts from susceptible (G88 and Wapio) and spinosad resistant (Pearl-Sel) strains of diamondback moth. Number boxes represent the exons of *Pxyl*α*6*. The inverted black triangles indicate the approximate location of the premature stop codons. White triangles indicate the location of the intron splice site variations with the sequence of the insert above the triangle. The frequency of each type of transcript in the G88, Wapio and Pearl-Sel strains is shown to the right of each diagram. Isoforms not seen in the G88, Wapio or Pearl-Sel strains as indicated in the figure were seen in clones from the F_2 backcross experiment (Table 3.5). Isoforms were numbered based on prior conventions (Grauso et al., 2002, Rinkevich and Scott, 2009) (isoforms I, III, XV, XVI) or arbitrarily (XXVI-XXX).

Table 3.3 Comparison of percent identity of the nucleotide and amino acid sequences and frequency of exons 3a, 3b, and 8b of the α6 subunit orthologs between *P. xylostella*, *B. mori* and *D. melanogaster*.

		Pxylα6 vs.		Frequency		
Exon		*Dα6*	*Bmα6*	G88	Wapio	Pearl-Sel[a]
3a	nt	66.7%	88.9%	0.09	1.00	0.47
	aa	93%	100%			
3b	nt	82.2%	95.6%	0.91	0.00	0.53
	aa	93%	100%			
8b	nt	75.9%	90.8%	1.00	1.00	1.00
	aa	100%	100%			

[a] the calculation of the frequency of 3a and 3b in Pearl-Sel does not include isoform XXX (Figure 3.3N) because isoform XXX does not possess exon 3.

Wapio. The nucleotide sequence of *Pxylα6* exon 3b is 82.2% and 95.6% identical compared to *Dα6* exon 3b and *Bmoα6* exon 3b, respectively. The translated amino acids coded by *Pxylα6* exon 3b are and 93% and 100% identical to both *Dα6* exon 3b and *Bmoα6* exon 3b, respectively. Exon 3b contains a serine phosphorylation site at amino acid position 76 that is absent in exon 3a. The frequency of exon 3b usage was 0.91 in G88 and 0.00 in Wapio.

There was only one exon 8 (8b) observed. The nucleotide sequence shares 75.9% and 90.8% identity with *Dα6* exon 8b and *Bmoα6* exon 8b, respectively. The translated amino acids coded by *Pxylα6* exon 8b are 100% identical to both *Dα6* and *Bmoα6* exon 8b.

A splice site variation of intron 10 (spliced out 30 bp upstream) was observed at a frequency of 0.16 in G88 and 0.00 in Wapio). This splice site variation added an additional 10 amino acids to the protein in middle of the intracellular linker between TM3 and TM4. These amino acids add two serine phosphorylation sites, putatively. This insertion was seen in isoforms I and III.

A-to-I RNA-editing was investigated at 16 previously identified RNA-editing sites (Tian et al., 2008). Putative A-to-I RNA-editing is summarized in Table 3.4. In the G88 strain, editing sites 1, 7, 9, 10, 11, 13, 14 and 15 were A in all samples, even though editing sites 1, 11, and 15 were edited in *Bmα6* (Jin et al., 2007). Editing sites 2, 3, 4b, and 8 had G in all clones. It is unknown whether these are the result of high frequency RNA-editing, or encoded G genomically as in the case of *Bmoα6*. Highly conserved editing sites 5, 6, and 12 were G at frequencies of 0.25, 0.79, and 0.80, respectively. These sites are edited at a high frequency in *Bmoα6* (Jin et al., 2007). Editing at site 16, which is edited only in *Amelα6* (Jones et al., 2006), was observed in 1 clone. A new putative editing site, arbitrarily named 4a, at position 391 was G

Table 3.4 Putative A-to-I RNA-editing sites in *Pxyl*α*6*. Editing site number corresponds to the convention set forth by Tian et al. (2008). Site 4a and 4b are designated arbitrarily. The position is numbered based on *Pxyl*α*6* nucleotide numbering of the open reading frame. The ratio in parenthesis indicates the proportion of clones that were edited at that site.

		Strain			
Site	Position	G88	Wapio	Pearl-Sel	*Bm*α*6* Counterpart
1	377	A	A	A	Edited
2	379	G	G	G	Genomic G
3	384	G	G	G	Genomic G
4a*	391	G (14/24)	A	A	Not Edited
4b	392	G	G	G	Edited
5	394	G (6/24)	A	G (19/27)	Edited
6	395	G (19/24)	A	G (19/27)	Edited
7†	400	A	A	A	Not Edited
8†	401	G	G	G	Genomic G
9†	430	A	A	A	Not Edited
10†	431	A	A	A	Not Edited
11	443	A	A	A	Edited
12	447	G (20/24)	G	G	Edited
13†	449	A	A	G (5/27)	Not Edited
14	451	A	A	A	Not Edited
15	454	A	A	A	Edited
16†	470	G (1/24)	A	A	Not Edited

* = New putative editing site
† = Edited in *A. mellifera* only

at a frequency of 0.58, resulting in an S131G substitution. In the Wapio strain, only editing sites 4b and 12 were edited. Both of these sites edited at a frequency of 1.0. No RNA editing was observed on exon 4 of *Pxylα*6, although it was found in *Bmoα6* (Jin et al., 2007). These putative editing sites need to be confirmed with genomic sequence information.

3.3.2 *Pxylα6* from the spinosad resistant Pearl-Sel strain

All transcripts from the spinosad resistant Pearl-Sel strain coded for apparently non-functional *α*6, and all transcripts contained premature stop codons, except isoform XXX (Figure 3.3B, E-N). Multiple isoforms were found. Isoforms Ib and IIIb have an intron 9 donor splice site variant that adds 40 additional bases (Figure 3.3B and D). These variants result in a premature stop codon at amino acid 341, which would produce a protein that is truncated shortly after TM3. Isoform XXVII lacks the intervening sequence between the first 20 bases of exon 4 through the first 16 bases of exon 9 (Figure 3.3J). This deletion results in a premature stop codon at amino acid 157 and would code for a protein with only loops D, A and E. Isoform XXVIIb is missing the intervening sequence between the first 20 bases of exon 4 through the first 16 bases of exon 9 and has the intron 9 donor splice site variant that add 40 bases (Figure 3.3K). Isoform XXX remains in frame, but is missing exons 3 through 9 (Figure 3.3N). This transcript would produce a protein without extracellular loops or TM1-3. Transcripts with premature stop codons or large gaps in critical sequences were not seen in the spinosad susceptible G88 or Wapio strain. The 3' splice site of intron 10 that was spliced out 30 bp upstream and adds 10 additional amino acids to the protein was seen in the Pearl-Sel strain at a 0.28 frequency. This insertion was limited to isoforms Ib and XXVIIb.

RNA-editing was generally similar between the Pearl-Sel and spinosad susceptible strains, although there were some differences. Editing sites 5 and 6 were both G at a frequency of 0.70, while editing site 12 was G in all samples. In the Pearl-Sel strain, editing site 13 was G at a 0.19 frequency. The same site was unedited in the G88 strain and only edited in *Amelα6* (Tian et al., 2008). Editing sites 4a and 16 were A in all Pearl-Sel samples.

3.3.3 F_2 Backcross and Bioassay

The genotypes of the backcross and bioassayed insects are summarized in Table 3.5. The parental genotypes were confirmed in that the G88 strain produced only full length transcripts and Pearl-Sel produced truncated transcripts. The F_1s showed close to the expected frequency of susceptible (f = 0.37 (isoforms I and III)) and resistant transcripts (f = 0.62 (isoforms IIIb, XVId, XXVII, and XXVIIb; Figure 3.3D, G, J, and K)). The bioassay survivors produced only resistant transcripts while those killed in the bioassay produced both susceptible (0.35 isoforms I and III), and resistant isoforms (0.64 isoforms IIIb, XV, XXVI, XXVIIb, XXIXb, and XXX; Figure 3.3D, F, I, K, M and N). This association of only truncated transcripts with the resistant phenotype suggests that *Pxyla6* is involved in spinosad resistance in the Pearl-Sel strain of diamondback moth.

3.4 Discussion

The full length cDNA of *Pxylα6* from spinosad susceptible strains codes for a typical nAChR subunit with an extracellular ACh binding domain and 4 transmembrane segments. The pattern of alternative exon use was similar to *Bmα6* (Jin et al., 2007), but was very different from *Dα6* (Grauso et al., 2002) and *Tcasα6* (Rinkevich and Scott, 2009). The presence of only two full

Table 3.5 Genotypes of diamondback moths used in the F_2 backcross-bioassay procedure. Isoforms I and III are full length or susceptible transcripts while the remaining isoforms are truncated or resistant transcripts.

		Isoform						
		Susceptible		Resistant				
Sample	n[a]	I	III	IIIb	XVId	XXVII	XXVIIb	Other[b]
Pearl Parental ♂	10(1)	0	0	10	0	0	0	0
G88 Parental ♀	14(2)	6	8	0	0	0	0	0
F_1	40(3)	7	9	8	6	2	8	0
BC_1 Untreated	15(3)	0	0	0	1	0	14	0
BC_1 Survivors	15(3)	0	0	7	0	0	8	0
BC_1 Dead	28(2)	0	10	7	0	0	7	4

[a]Number in parentheses indicates how many independent batches of larvae were used.
[b]Other isoforms include one sample of isoforms XV, XXVI, XXVIIIb, and XXIX.

length isoforms (I and III) is the lowest number observed in any insect thus far, although *Bmoα6* has only I, III, IV (also named Type III in (Jin et al., 2007)) and XVI (Shao et al., 2007). Only one exon 8 (8b) was found for *Pxylα6*, consistent with results in *Bmoα6* (Jin et al., 2007). The lack of exon 8a and 8c in *Pxylα6* transcripts supports the observation that exon 8a was lost and exon 8c is not transcribed in the Lepidoptera lineage (Jin et al., 2007, Baxter et al., 2010). No optional exon use was observed in *Pxylα6* transcripts from susceptible strains. Thus, it appears that Lepidoptera produce a relatively small number of splicing isoforms.

Receptor function may be affected by the additional 10 amino acids coded by the intron 10 splice site variant that adds two putative phosphorylation sites and four additional hydrophobic residues (GenBank Accession GQ247883). Alanine-scanning and chimera construction showed that hydrophobic residues in the TM3-TM4 intracellular domain are important for subunit expression, surface manifestation and toxin binding (Ren et al., 2005, Kracun et al., 2008). These amino acids are added after the early stretch of highly conserved amino acids immediately following the TM3 segment. Residues following the conserved sequence tend to be highly variable across subunits and species (Jones and Sattelle, 2007). Phosphorylation at the intracellular linker between TM3 and 4 is important for receptor desensitization and conductance (Huganir et al., 1986, Thany et al., 2006). Interestingly, a similar 3' splice site variant was seen in *Amelα4* where 2 phosphorylation sites were added to the intracellular linker between TM3 and TM4 (Jones et al., 2006). This similarity between divergent organisms indicates 3' splice site variation that introduces post-translational modification sites may be important for generating pharmacologically unique proteins. Given that this splice variant was found in both G88 and Pearl-Sel indicates it is not associated with spinosad resistance. This splice variant was not previously detected (Baxter et al., 2010).

Putative A-to-I RNA-editing occurs at 5 sites on exon 5 of *Pxylα6*. I observed a putative novel editing site (4a, resulting in a S131G substitution), consistent with a previous report (Baxter et al., 2010). Substituting serine with glycine at this position may affect protein folding or receptor function as it occurs in loop E, which is important for agonist binding (Amiri et al., 2008). This putative editing site was only seen in G88, but not Wapio or Pearl-Sel, suggesting it is not involved in spinosad resistance.

No *Pxylα6* transcripts from the Pearl-Sel strain code for complete proteins. I detected many novel isoforms (IIId, XVId, XVIe, XXVII, XXVIIb, XXVIIIb, XXIX, and XXX) that have not been previously reported. I did not detect isoforms I/IIIc, or XXV that were previously reported (Baxter et al., 2010). The proteins coded for by transcripts with premature stop codons are illustrated in Figure 3.4. These novel isoforms were overlooked in a previous report because the primers used in PCR were for exons 7-12 (Baxter et al., 2010). Thus, this technique was unable to record the skipping of exon 3 (isoforms XVId and XVIe; Figure 3.3G and H) or exon 5 (isoforms XXVIIIb and XXIX; Figure 3.3L and M), the large missing regions between exons 4 through 9 (isoforms XXVII and XXVIIb; Figure 3J and K), or the 7 bp addition at the beginning of exon 4 (isoforms IIId, XVId, and XVIe; Figure 3.3E, G, and H). I feel identification of these isoforms is of significant importance because in Pearl-Sel isoforms IIId and XXVIIb (Figure 3.3E and K) account for a majority of transcripts. More than half of the transcripts in bioassay

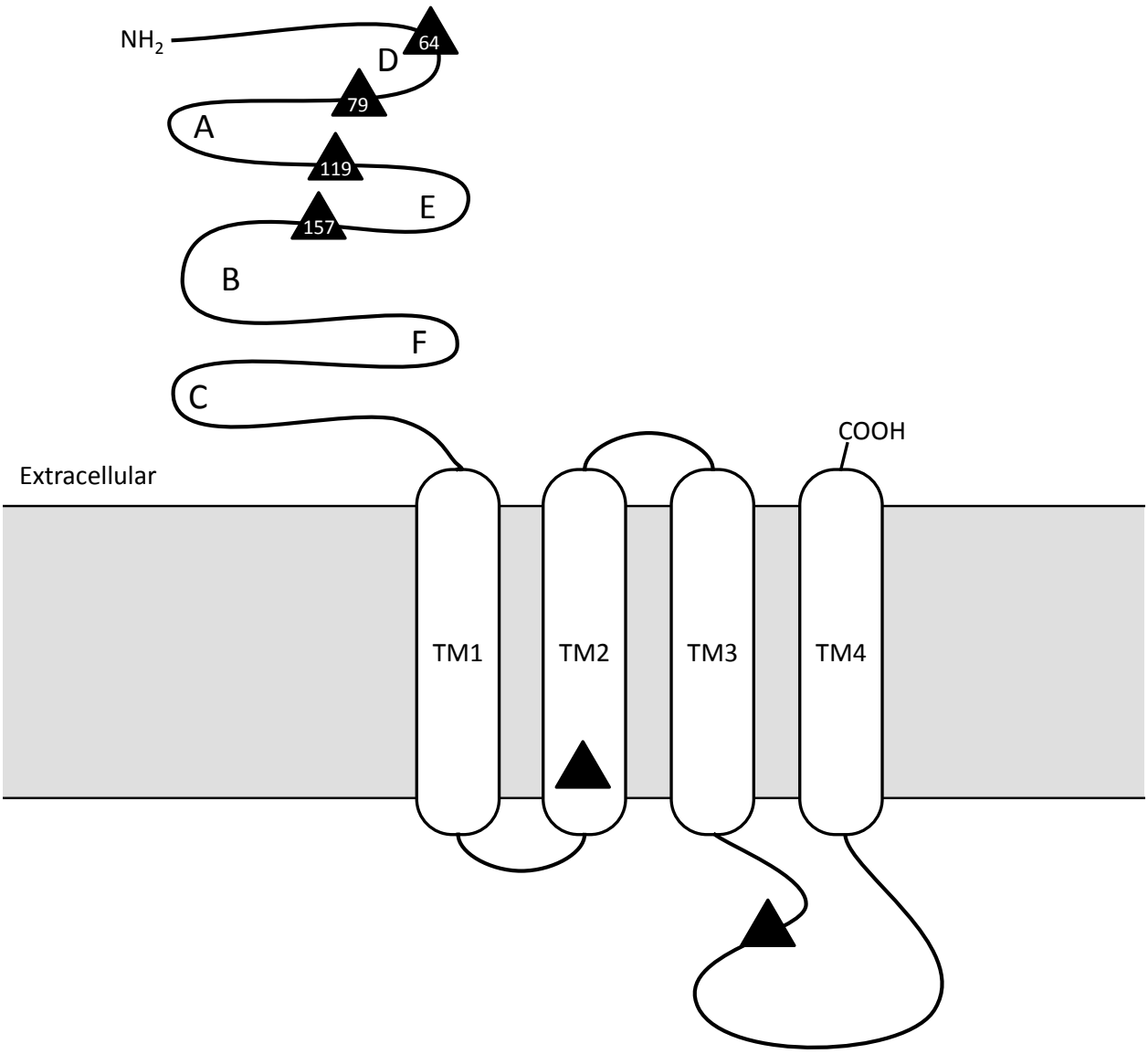

Figure 3.4 Diagram of the topology of Pxylα6. Ligand-binding loops are designated by letters in the extracellular portion of the protein. Transmembrane domains (TM1-4) are designated by cylinders. The amino acid positions of stop codons created by alternative splicing are shown by black triangles. The numbers inside the black triangles indicate which amino acid position the protein is truncated. The black triangles correspond to the position indicated by black triangles in Figure 3.3.

survivors are isoform XXVIIb (Table 3.5). These isoforms would have earlier stop codons than those generated by isoforms Ib and IIIb. In the case of isoforms IIId, XVId, and XVIe, transcripts would produce a protein with only loop D, which, most likely, is a non-functional protein at any level. The proteins created by transcripts with premature stop codons are displayed in Figure 4.

The mechanism(s) responsible for generating transcripts with premature stop codons is unknown. The appearance of isoforms in the F_1s and backcross that were not observed in the parental strains suggests truncated transcripts are not produced in a simple Mendelian fashion. Based on the observed parental transcripts, the F_1s should have only produced isoforms I, III, and IIIb. Unexpectedly, isoforms XXVII, XXVIIb, and XVId were seen in the F_1s. Isoforms XV, XXVI, XXIXb, and XXX were only seen in the BC dead sample. These results suggest that the truncated isoforms are generated by differential splicing through multiple resistance alleles or altered splicing machinery is responsible for the errant splicing. These transcripts may also be tissue-specific splice variants. However, little is known about the distribution of nAChR expression in insects (Sattelle et al., 2005), let alone tissue-specific splice variation.

The recessive pattern of spinosad resistance in the Pearl-Sel strain (Zhao et al., 2002) is consistent with the observation of only truncated transcripts in bioassay survivors and both full length and truncated transcripts in animals killed by the bioassay. Transcripts of nAChRs with intron 3′ splice site variations and transcripts with premature stop codons have been reported in other insects (Rinkevich and Scott, 2009, Gao et al., 2007c). In *Amelα3*, a truncated transcript was seen that was similar to the *Pxylα6* transcript that possessed an intron 9 3′ splice site variant in that they both code for proteins that terminate between TM3 and TM4 (Jones et al., 2006). Little information exists about the *in vivo* function of these truncated proteins. *In vitro* receptors formed by truncated Dα1 transcripts do not produce currents when acetylcholine is bound

(Schulz et al., 2000). Interestingly, truncating *Dβ1* transcripts via EMS mutagenesis produces reduces sensitivity to neonicotinoids (Perry et al., 2008). While this is an interesting prospect for a spinosad and/or imidacloprid resistance mechanism via truncated *Pxylα6* transcripts, neonicotinoids and spinosad interact with different areas and types of receptor complexes (Salgado and Saar, 2004, Orr et al., 2009).

While the work in diamondback moth may serve as a foundation for future studies of spinosad resistance in other insects, truncated transcripts as a mechanism of spinosad resistance could be unique to diamondback moth. It would be of interest to study *Pxylα6* in the Field CH1 strain of diamondback moth as spinosad resistance in that strain shares a similar pattern of inheritance and is unable to be overcome with metabolic synergists (Sayyed et al., 2004). The Spino-SEL strain of diamondback moth relies on cytochrome-P450 monooxygenases and esterases as a major mechanism of spinosad resistance (Sayyed et al., 2008). Therefore, truncated transcripts of *Pxylα6* may not be important in Spino-SEL. Although spinosad resistance in the housefly shares a similar pattern of inheritance and is unable to be reduced with synergists (Shono and Scott, 2003), spinosad resistance is not due to mis-spliced *Mdα6* (Gao et al., 2007c), *Mdα5 or Mdβ3* (Gao et al., 2007b). The incongruence of spinosad resistance in housefly with these results in diamondback moth suggests I should be cautious of extrapolating truncated transcripts as a resistance mechanism to other species. It will be interesting to elucidate the expression of *α6* orthologs in spinosad resistant tobacco budworm (Wyss et al., 2003) and western flower thrips (Bielza et al., 2007) in order to determine if mis-spliced *α6* orthologs are a widespread spinosad resistance mechanism.

3.5 Acknowledgements

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Chapter 4

Transcriptional diversity and allelic variation in nicotinic acetylcholine receptor subunits of the red flour beetle, *Tribolium castaneum*[2]

4.1 Introduction

Nicotinic acetylcholine receptors (nAChRs) are cationic selective members of the cys-loop ligand-gated ion channel superfamily. They facilitate rapid cholinergic transmission in the insect CNS by binding acetylcholine (ACh). Receptor complexes in insects can be heteropentamers consisting of two α and three β subunits (Thany et al., 2006) or homopentamers of only α subunits (Fayyazuddin et al., 2006). The α subunits are defined by a characteristic YxCC motif in the ACh-binding extracellular loop C, whereas β subunits lack this feature (Karlin, 2002). Receptor subunits consist of four transmembrane segments (TM1-4) and an extracellular ligand binding domain (loops A-F) at the N-terminus (Karlin, 2002). The second transmembrane domain lines the pore of the receptor complex (Imoto et al., 1988). Hydrophobic amino acids in the intracellular linker between the third and fourth transmembrane domain are important for cell surface expression of certain subunits (Ren et al., 2005).

The nAChRs are a diverse family of ion channels with many subunits found in every multicellular animal species. In insects, there are 12 subunit genes in *Bombyx mori* (Shao et al., 2007) and *Tribolium castaneum* (Jones et al., 2007), 11 in *Apis mellifera* (Jones et al., 2006), and 10 in *Anopheles gambiae* (Jones et al., 2005) and *Drosophila melanogaster* (Sattelle et al., 2005). Transcripts of these genes, especially α6 subunit orthologs, may be modified by alternative exon usage and/or A-to-I RNA editing (Sattelle et al., 2005, Jin et al., 2007). In *D. melanogaster*, more than 30,000 transcriptional variants of *Dα6* may be produced from combinations of alternative exon usage and A-to-I RNA editing (Grauso et al., 2002).

[2]Previously published as Rinkevich, F.D., and J.G. Scott. (2009) Insect Molec. Biol. 18, 233-242

Transcriptional diversity of orthologous *α6* subunits is conserved across widely divergent insects (Jin et al., 2007). Alternative exon use and A-to-I RNA editing may affect subunit coassembly (Lansdell and Millar, 2000) and agonist-induced currents (Saragoza et al., 2003). A deficit in RNA editing is the underlying cause of amyotrophic lateral sclerosis (Kawahara et al., 2004).

Transcripts containing one or more introns have been reported from a number of α subunits from insects (Grauso et al., 2002, Jones et al., 2006, Jones et al., 2005, Gao et al., 2007c). In each of these cases, this leads to a stop codon being introduced, which eliminates crucial motifs such as transmembrane segments and ligand-binding domains that are required for proper receptor assembly and function. Despite the loss of these critical motifs caused by unspliced introns, the fact they have been observed in multiple α subunits from highly divergent insects suggests a possible functional role for these truncated transcripts. It is unknown if these transcripts are subject to nonsense mediated decay (Chang *et al*., 2007). Although no physiological studies have identified a role for these truncated transcripts in insects, it has been suggested they code for proteins that may moderate synaptic events in a similar manner to ACh-binding protein in *Lymnaea stagnalis* (Sattelle et al., 2005) or α7 in mice (Saragoza et al., 2003).

Spinosad and imidacloprid are two commercially important insecticides (a macrocyclic lactone and neonicotinoid, respectively (Tomizawa and Casida, 2005, Nauen and Denholm, 2005)). Both insecticides act primarily on the nAChR, but on different types of nAChRs (Salgado and Saar, 2004). Studies on a lab-selected, imidacloprid resistant strain of the brown plant hopper (*Nilaparvarta lugens*) indicated that the highly conserved tyrosine in extracellular loop B of Nlα1 and Nlα3 was important for imidacloprid binding and toxicity as well as inheritance of resistance (Liu et al., 2005). EMS-mutagenized *Drosophila* with various mutations in *Dα1* or *Dβ2* all resulted in strains with resistance to imidacloprid and other neonicotinoids

(Perry et al., 2008). A *pogoR11* element-mediated knock-out mutation in *Dα6* where the protein is truncated after TM2, resulted in 1181-fold resistance to spinosad (Perry et al., 2007). Spinosad resistant houseflies derived from field collected resistant populations, however, showed no differences in the coding sequences, post-transcriptional modifications, or levels of *Mdα6* transcripts compared to a genetically related susceptible strain (Gao et al., 2007c). As such, the mechanism(s) of spinosad resistance remains unknown.

The genome of *T. castaneum* has recently been published (Consortium, 2008) and 12 nAChR subunit genes were identified (Jones et al., 2007). *T. castaneum* is an ideal study organism because it is easy to rear, its genetics and genome are well understood, and it is easy to manipulate with molecular techniques such as RNAi, gene-knockouts, and germ-line transformation (Consortium, 2008). Besides being an ideal model organism, *T. castaneum* is also a serious pest of stored products causing more than $1 billion (US) in damage annually (Throne et al., 2003). While sequences of nAChR subunit gene transcripts have been reported (Jones and Sattelle, 2007), the presence of additional transcript variations that have been reported in other subunits from other animals (Grauso et al., 2002, Jones et al., 2005, Jin et al., 2007, Gao et al., 2007c) have not been investigated. Applying similar approaches as Jones *et al.* but using a more genetically diverse strain may yield novel transcripts and alleles. Spinosad and imidacloprid are promising control agents against *T. castaneum* (Toews et al., 2003). Since both compounds act on nAChRs, it would be prudent to make use of genomic information as a guide to help provide the ground work for understanding the role of nAChR subunits in the toxicity of spinosad and imidacloprid. Herein, I report the full length sequences for the open reading frames of all 12 nAChR subunit cDNAs from *T. castaneum*, compare my findings to previous results (Jones and

Sattelle, 2007), and identify other novel transcripts to establish a solid framework for future studies on nAChRs in *T. castaneum*.

4.2 Materials and Methods

4.2.1 Beetle Rearing

The wild-type, insecticide susceptible GA-1 strain of red flour beetles (*Tribolium castaneum*) was obtained from Kathy Leonard at Kansas State Univ. The GA-1 strain has been reared in the laboratory for more than 28 years without exposure to insecticides. Beetles were reared in 475 ml mason jars (Kerr, Jackson TN) with approximately 75 g of organic golden buffalo flour (Heartland Mill, Merienthal KS) with 5% (wt/wt) of dried baker's yeast (MP Biomedicals, Solon OH). The top of the jar was covered with a fine mesh cloth and sealed with a canning band. Jars were held at 30^{o}C with 65% $\pm$ 5% humidity in continuous darkness. Adult beetles were transferred every four weeks to new rearing jars using the paper transfer method with a strip of paper towel (USDA, 2008 http://bru.gmprc.ksu.edu/proj/tribolium/wrangle.asp). Three colony jars were used each transfer.

4.2.2 RNA Isolation

Total RNA was isolated from 5, unsexed, 1-5 day old adults using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Total RNA was resuspended in 100 μl of DEPC treated H_2O. RNA concentration was measured on a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE) by dividing the absorbance at 260 nm by the specific absorption coefficient of RNA (Gallagher and Desjardins, 2006).

4.2.3 Reverse Transcription, PCR, Cloning and Sequencing

Reverse transcription (RT) was performed using 5 μg of total RNA using SuperScript III according to the manufacturer's directions (Invitrogen, Carlsbad, CA) with the reverse transcription time extended to 3 hrs. Two μl of cDNA were used in PCR to amplify specific subunits (Table 4.1) using 100 μl volumes and GoTaq Green Master Mix (Promega, Madison, WI). PCR products were visualized on 1% agarose gels stained with 50 μg/ml ethidium bromide under UV light. PCR products were directly cloned into pGEM-T Easy cloning vector (Promega, Madison, WI) according to the manufacturer's directions for chemically competent cells except the ligation step was performed at 4^{o}C >12 hrs. Individual colonies were screened for the presence of an insert using the colony as a template in PCR. Colony PCR was conducted in 15 μl volumes using GoTaq and T7 and SP6 primers. PCR products were visualized on 1% agarose gels and positive colonies were incubated at 37^{o}C overnight in 3 ml of LB medium containing 100 μg/ml ampicillin. Plasmid DNA was isolated using the Wizard Plus SV Minipreps (Promega, Madison, WI) and eluted in 100 μl H_2O. Positive clones were sequenced from both ends using T7 and SP6 primers as well as an internal gene specific primer (Table 4.1) at Cornell's Biotechnology Resource Center. Sequences for each subunit were aligned using MegAlign (DNA Star, Madison WI) in order to determine the full-length sequence of each subunit was determined. The electropherograms were manually examined for clones if sequences differed from each other or those previously reported. A minimum of 10 clones were sequenced for each subunit. If less than 2 clones had an identical substitution, this nucleotide difference was assumed to be a Taq error which happens with a frequency of about 10^{-4} (Chen et al., 1991, Eckert and Kunkel, 1990). If two or more clones shared a nucleotide difference, they were

Table 4.1 Sequences of primers used.

Subunit	Primer Name	Sequence
Tcasα1	Tcasa1FullF	ATGGAGCTGTTGCTCGCCCTTTCATGGTT
	Tcasa1FullR	TTACAACTCCTCCGGTACCATTTTCAAC
	Tcasa1IF	CGGTAACTACGAAGTGACCA
Tcasα2	Tcasa2FullF	ATGTGGTGGTGGCTGAATTTCGG
	Tcasa2FullR	TTAGAAATCTAATTCAGGTAGAAATTG
	Tcasa2IF	TTCGGACTGTTTTTGTCGG
Tcasα3	Tcasa3FullF	ATGAAGAGCTTGGTGGGGATTATGTGG
	Tcasa3FullR	TTATAAAGAACTGGCAATATGGGGTTTAGC
	Tcasa3IF	TGTGGATAGTTTTAGTGCTC
Tcasα4	Tcasa4FullF	ATGCCTCCTTCTGCCGCCGAGACGCT
	Tcasa4FullR	CTACTGTGGAGGAGGGCAACGAACAAC
	Tcasa4IF	GAGACGCTGAGAGCCTG
Tcasα5	Tcasa5FullF	ATGAAACTGTTTTTGATAGTTGTAATCC
	Tcasa5FullR	CTAAGAGGCCGCGTCGTTTGCCGGACTTC
	Tcasa5IF	CAGTCATCTGTAATTAACACG
	Tcasa5Exon78F	CCTCGTAGTTGATCTCAATC
	Tcasa5Exon10R	CTGAAGCCTACTCGAGCT
	Tcasa5Exon8F	TTGATCTCAATCGCAAATTTCT
Tcasα6	Tcasa6FullF	ATGGTCCGGAGCGAGCA
	Tcasa6FullR	CTACTGCACGATTATGTGC
	Tcasa6IF	TGGTGCTCAATTCCGAATC
Tcasα7	Tcasa7FullF	ATGGACAGTGGCATGCAGCAATGTC
	Tcasa7FullR	CTACGATACGATGATGTGAGGTGCCG
	Tcasa7IF	GATATCAGCAGCTTTATAAC
Tcasα8	Tcasa8FullF	ATGTACTTGTTAAAGTTTACATGTTG
	Tcasa8FullR	TTACAACCTCAAAGCGCCCCTATC
	Tcasa8IF	GTCATCTGGAAGCCTCCTTC
Tcasα9	Tcasa9FullF	ATGGGCATTCTAAATTATTTTCTCCCCTTTTTGC
	Tcasa9FullR	CTAAGGGGTGAACCCGATAATCATCCCCAA
	Tcasa9IF	CGACCTCTCCGTCTACAAC
Tcasα10	Tcasa10FullF	ATGGCAAACATACTGAGGCTCGTGGTTG
	Tcasa10FullR	TCACACGGAATAAACTATGGTTAAAATGAG

	Tcasa10IF	CTTGGCCGATCACGAAATTTGGC
Tcasα11	Tcasa11FullF	ATGTATTTAATAAAATTAGTTTGTTTCAC
	Tcasa11FullR	TTATTGTGTGGGGATGTGTGTGCGCGA
	Tcasa11IF	GTAATTTGGAAGCCACCTTCG
Tcasβ11	TcasBFullF	ATGGAAGAAAGCAAATGTTCGGAG
	TcasBFullR	TTATTTGCCGCGGTAAATTTCG
	TcasBIF	TACCCCAATGGCGAGGTGTTGTG
pGEM	T7	TAATACGACTCACTATAGGG
Vector	SP6	TATTTAGGTGACACTATAG

considered to be a polymorphism. A-to-I RNA-editing was evaluated at sites previously shown to be edited in *T. castaneum* (Jones and Sattelle, 2007), *A. mellifera* (Jones et al., 2006), *B. mori* (Shao et al., 2007), *D. melanogaster* (Grauso et al., 2002, Hoopengardner et al., 2003), and *M. domestica* (Gao et al., 2007c, Gao et al., 2007b).

4.3 Results

4.3.1 Cloning Summary

All 12 nAChR subunit full length cDNAs, including transcripts with numerous post-transcriptional modifications were successfully cloned and sequenced using reverse transcription followed by PCR based on published sequences. All subunits except *Tcasα1* contained novel alleles or transcriptional modifications distinct from those previously reported (Jones and Sattelle, 2007). The cloning results are summarized in Table 4.2. GenBank accession numbers for all transcripts and alleles are listed in Table 4.3.

4.3.2 Alternative Exon Usage

Alternative exon use was only found in *Tcasα4* and *Tcasα6*. The nucleotide sequence of alternative exons 4 and 4′ of *Tcasα4* were identical to what was previously reported (Jones and Sattelle, 2007). Exon 4 of *Tcasα4* codes for loops E and B as well as the cys-loop, which are important for ligand binding (Shimomura et al., 2005). Alternative exons were observed for exons 3 and 8 of *Tcasα6* as previously reported (Jones and Sattelle, 2007), except exon 8c was not detected in any transcripts (n=39). I found a novel exon 8d in 1 of the 39 clones. While exons 8a, 8b, and 8c share 55-63% nucleotide and 65-79% amino acid similarity, exon 8d shared only

Table 4.2 Variation in *T. castaneum* nAChR cDNAs. Modifications with an asterisk indicate modifications that are seen in all clones that were sequenced. Underlined modifications indicate previously reported post-transcriptional modifications. The number in parentheses following the number of alleles indicates the number of polymorphisms among alleles for that subunit.

Subunit	n†	Modifications
Tcasα1	12	None
Tcasα2	10	6 alleles (7), intron 3 splice variant
Tcasα3	11	2 alleles (1), unspliced introns 9 and 10, intron 4 splice variant
Tcasα4	15	2 alleles (1), alternative exons, unspliced intron 7
Tcasα5	11	3 alleles (8), Δexons $^{8\text{-}10}$*
Tcasα6	39	2 alleles (1), alternative exons‡, RNA-editing, Δexons^{3,4,5,6,7,8}, splice variants of introns 5 and 7
Tcasα7	10	2 alleles (1)
Tcasα8	10	5 alleles (13), intron 1 splice variant
Tcasα9	11	5 alleles (6), unspliced introns 5 + 6
Tcasα10	12	Unspliced introns 2 + 3, intron 5 splice variant
Tcasα11	10	2 alleles (10), unspliced intron 6
Tcasβ1	18	2 alleles (3), unspliced intron 6

† = total number of clones sequenced
‡ = see Figure 4.2
* = seen in all clones sequenced

Table 4.3 GenBank accession numbers for transcripts and alleles described in the text.

Subunit	Description	n[a]	Accession Number
Tcas α2	Intron 3, 3′ splice site variant	1	EU926747
	Allele v1	5	EF526081
	Allele v2	1	EU926748
	Allele v3	1	EU930051
	Allele v4	1	EU930052
	Allele v5	1	EU930053
	Allele v6	1	EU930054
Tcas α3	Allele v1	4	EF526082
	Allele v2	7	EU930049
	Intron 4, 3′ splice site variant	2	EU930056
	Unspliced introns 9 and 10	1	EU930055
Tcas α4	Allele v1	6	EF526083
	Allele v2	9	EU930057
	Unspliced intron 7	1	EU930058
Tcas α5	Allele v1	2	EF526085
	Allele v2	3	EU930050
	Allele v3	6	EU930059
	Deletion of exons 8-10	11	EU930060
Tcas α6	Allele v1	37	EF526086
	Allele v2	2	EU930061
	Intron 5, 3′ splice site variant	1	EU930062
	Intron 7, 3′ splice site variant	1	EU930063
	Isoform I	5	EU930064
	Isoform II	9	EU930065
	Isoform III	7	EU930066
	Isoform IV	1	EU930067
	Isoform VI	2	EU930068
	Isoform XII	2	EU930069
	Isoform XIII	2	EU937784
	Isoform XIV	1	EU937785
	Isoform XV	1	EU937786
	Isoform XVI	1	EU937787
	Isoform XVII	1	EU937788
	Isoform XVIII	1	EU937789
	Isoform XIX	1	EU937790
	Isoform XX	1	EU937791
	Isoform XXI	1	EU937792
	Isoform XXII	1	EU937793
	Isoform XXIII	1	EU937794

	Isoform XXIV	1	EU937795
Tcasα7	Allele v1	1	EF526089
	Allele v2	9	EU937796
Tcasα8	Intron 1, 3′ splice site variant	1	EU937797
	Allele v1	4	EF526090
	Allele v2	2	EU937798
	Allele v3	2	EU937799
	Allele v4	1	EU937800
	Allele v5	1	EU937801
Tcasα9	Unspliced introns 5 and 6	1	EU937802
	Allele v1	7	EU937803
	Allele v2	1	EF526091
	Allele v3	1	EU937804
	Allele v4	1	EU937805
	Allele v5	1	EU937806
Tcasα10	Unspliced introns 2 and 3	1	EU937807
	Intron 5, 3′ splice site variant	1	EU937808
Tcasα11	Allele v1	5	EF526093
	Allele v2	5	EU937809
	Unspliced intron 6	2	EU937810
Tcasβ1	Allele v1	5	EF526094
	Allele v2	5	EU937811
	Unspliced intron 6	2	EU937812

a = number of clones observed with this sequence

21-25% nucleotide and 10-14% amino acid similarity with exons 8a, 8b, and 8c. The nucleotide sequence of exon 8d is 31 bp longer than exons 8a, 8b and 8c which are all 88 bp in length. Based on the published *T. castaneum* genome sequence (Consortium, 2008), exon 8d is separated from exon 8c by 982 bp, while 8a and 8b are separated by 337 bp and 8b and 8c are separated by 544 bp. The sequence of exon 8d indicates its first codon would be a stop codon, thus truncating the protein at this point (Figure 4.1).

For *Tcasα6,* I detected previously identified isoforms I through IV, but isoform V (Grauso et al., 2002) was not found. In all, 18 unique exon combinations and 14 novel isoforms were identified for *Tcasα6* (Figure 4.2). Five of 39 clones did not contain exon 3, ten clones did not contain exon 5 and five clones did not contain exon 8. Eight of ten clones missing exon 5 also had exon 3b. The two other clones missing exon 5 had exon 3a or 3x (no exon 3). A full description of each *Tcasα6* transcript, including schematic diagrams, frequency of observed exon combinations, and frequency of alternative exon usage is shown in Figure 4.2 and Table 4.4, respectively.

Previously reported exons 8, 9 and 10 for *Tcasα5* (Jones and Sattelle, 2007) were not found in any of the 11 clones that were sequenced. The absence of these exons did not affect the open reading frame of *Tcasα5* and increased the similarity to *α5* orthologs in *D. melanogaster* (+8.2%, (Grauso et al., 2002) and *A. gambiae* (+2.5%, (Jones et al., 2005), but decreased similarity to *A. mellifera* (-0.9%, (Jones et al., 2006). To verify that these previously reported exons were not present, primers were designed based on the sequence of previously reported exons 8, 9, and 10 and used in combination with primers used for full length amplification and sequencing of *Tcasα5* (Figure 3.3A). Primers sets specific for exons 3 + 12 (primers Tcasa5IF and Tcasa5FullR), or 8 + 10 (Tcasa5Exon8F and Tcasa5Exon10R) successfully amplified their

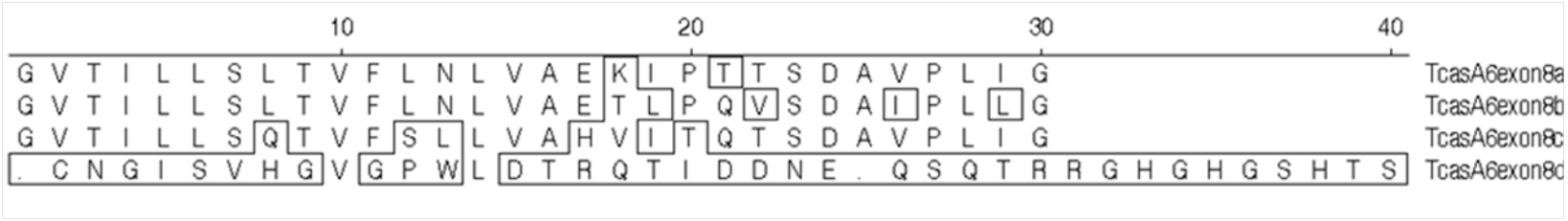

Figure 4.1 Alignment of deduced amino acids from exons 8a, b, c, and d of *Tcasa6*. Amino acids divergent from the consensus are boxed. Exon 8d shows <14% amino acid similarity to the other exons and introduces multiple stop codons, that are indicated by a period.

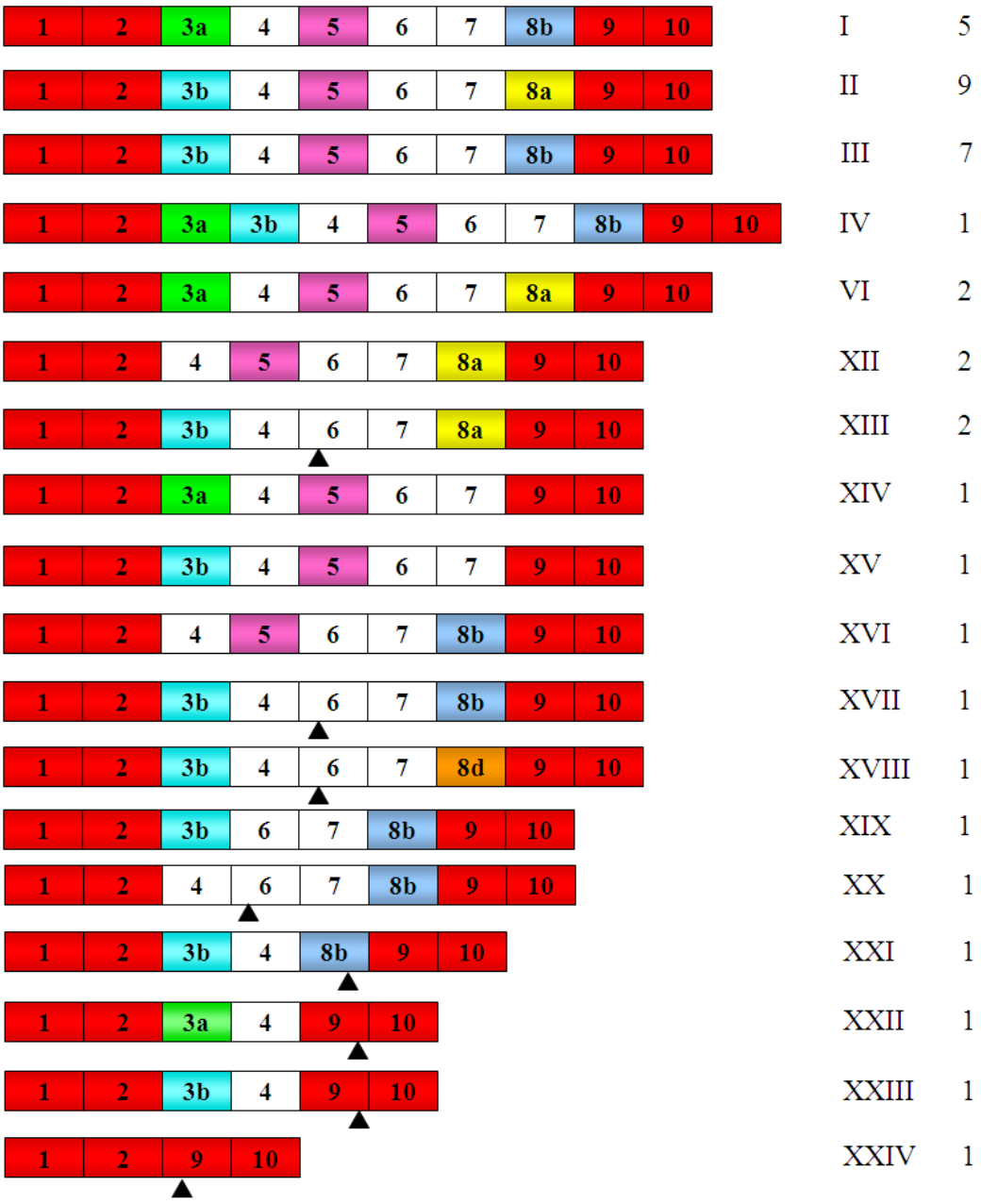

Figure 4.2 Schematic diagrams and frequency of variable exon isoforms in *Tcasα6*. Numbered boxes indicate exons found in that particular isoform. Conserved exons found in every isoform are colored red. Alternative exons 3a, 3b, 8a, 8b, and 8d are colored green, light blue, yellow, dark blue, and orange, respectively. Optional exon 5 is colored pink. Isoform numbering follows the convention put forth by Grauso *et al.* (isoforms I-V (Grauso et al., 2002)) and Gao *et al.* (isoforms VI-XI (Gao et al., 2007c)). Isoforms V and VII-XI are not listed because they were not detected. Isoforms XII through XXIV are arbitrarily numbered. The sizes of the exon boxes are not proportional to their nucleotide length. Triangles below exons indicate the approximate location of the introduction of a stop codon, due to a frame shift resulting from the loss of one or more exons.

Table 4.4. Exon usage frequency in *Tcasα6* transcripts (n = 39).

Exon	Frequency
3a	0.23
3b	0.61
3ab	0.03
3x	0.13
4	0.95
4x	0.05
5	0.74
5x	0.26
6	0.90
6x	0.10
7	0.90
7x	0.10
8a	0.38
8b	0.46
8d	0.03
8x	0.13

x = exon not used in transcript
Exon 8c was not observed in any clones that were sequenced.

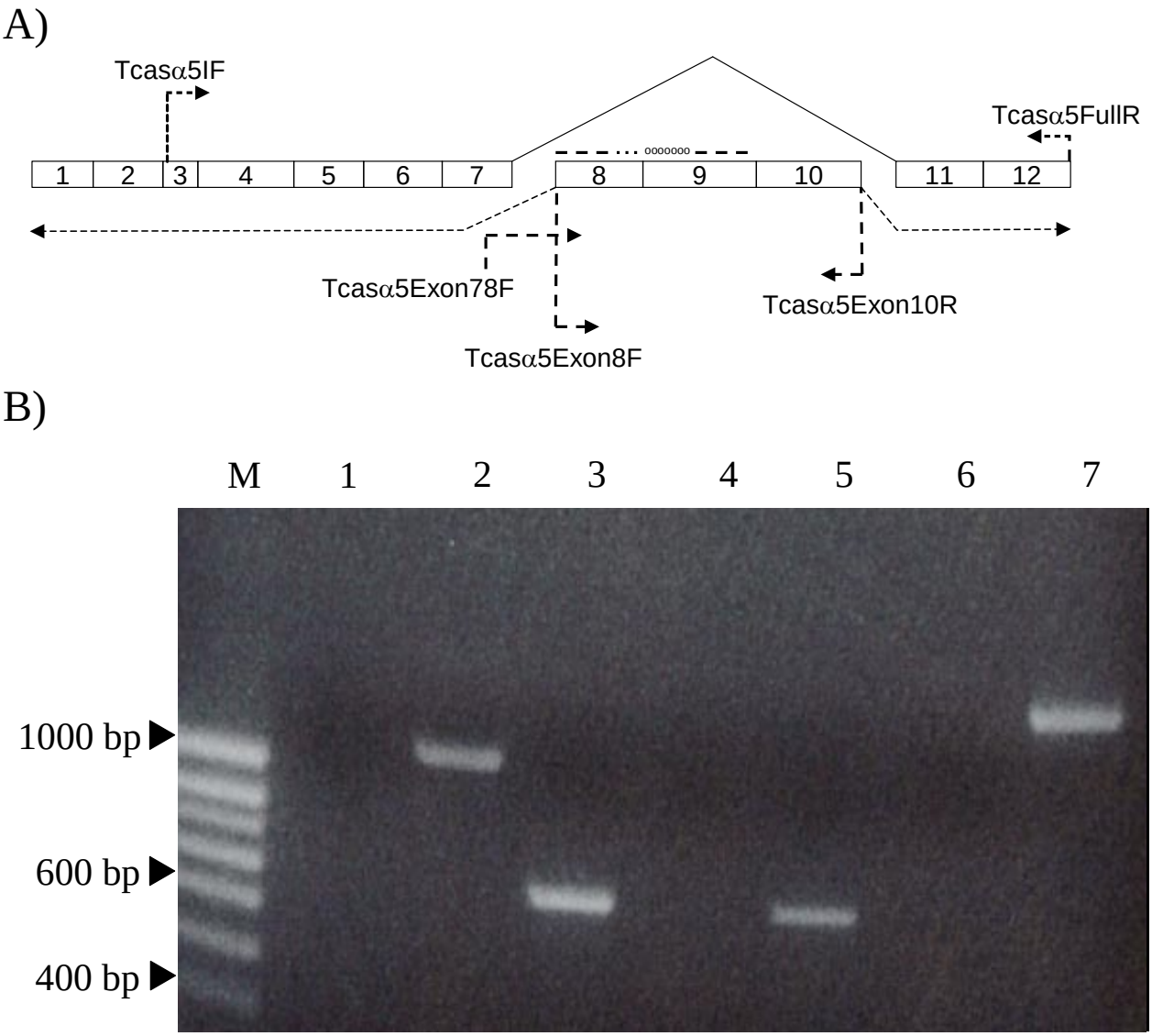

Figure 4.3 Schematic diagram (A) and gel photo of PCR products (B) used to validate exons 8-10 of *Tcasα5* were missing. (A) Tcasα5Exon78F spans the ends of exons 7 and 8. Solid lines over exons 8 and 9 represent the sequence of 141 identical bases shared between both exons. The dashed line over exon 8 represents the sequence of 43 identical bases shared between exon 8 and the 5′ end of intron 9. The circles over exon 9 represents the sequence of 93 identical bases shared between exon 9 and the 3′ end of intron 7. (B) The banding patterns show that previously reported exons 7, 8, and 9 are not included in transcripts containing other exons used in other full-length transcripts. Lane M) 5 μL HyperLadder IV (Bioline, Taunton, MA), 1) Tcasα5IF/ Tcasα5Exon10R, 2) Tcasα5IF/ Tcasα5FullR, 3) Tcasα5Exon78F/Tcasα5Exon10R, 4) Tcasα5Exon78F/ Tcasα5FullR, 5) Tcasα5Exon8F/ Tcasα5Exon10R, 6) Tcasα5Exon8F/ Tcasα5FullR and 7) Tcasβ1FullF/ Tcasβ1FullR as a positive control.

intended targets (Figure 4.3B, lanes 2 and 5). However, primer sets specific for exons 3 + 10 (Tcasa5IF and Tcasa5Exon10R), or 8 + 12 (Tcasa5Exon8F and Tcasa5FullR) failed to generate a PCR product (Figure 4.3B, lanes 1 and 6). The fragment generated by primers Tcasa5Exon78F (spanning exons 7 and 8) and Tcasa5Exon10R (exon 10 (Figure 4.3B, lane 3)) was cloned and sequenced. The sequence showed that primer Tcasa5Exon78F actually annealed between bases 86 to 106 of exon 9. Tcasa5Exon8F also annealed to exon 9 at positions 94 to 114 although it was specifically designed for the beginning of exon 8. The *T. castaneum* genomic sequence shows that exons 8 and 9 share an identical sequence of 141 bp. Exon 9 has an additional 93 bp on the 5′ end of the alignment overlap between these two exons while exon 8 extends 43 bp at the 3′ end. The 93 bp 5′ extension of exon 9 overlaps with 100% identity to the 3′ end of intron 7 and the 43 bp 3′ extension of exon 8 shares the exact same sequence as the 5′ end of intron 9. This explains why PCR products of the observed sizes were produced and demonstrates that previously reported exons 8 through 10 are likely not included in transcripts containing other exons in this strain. The results in Figure 4.3 show that previously reported exons 8, 9 and 10 are not found on transcripts containing other *Tcasα5* exons, but that there is a transcript containing only exons 8-10. The previously reported intron between exons 9 and 10 is not spliced out but remains in this transcript. It does not appear this is an alternatively spliced intron due to the presence of a single band on the gel. The function of this transcript is unknown.

4.3.3 Intron 3′ Splice Site Variation

Splice variants at the 3′ splice sites of introns were observed in *Tcasα2, α3, α6, α8,* and *α10*. Most splice variants resulted in the introduction of a premature stop codon. Intron 4 of *Tcasα3* was spliced out 10 bp upstream in two clones (out of 11) and lead to the introduction of a

premature stop codon. Introns 5 and 7 in *Tcasα6* were spliced out 179 and 91 bases upstream, respectively, in 2 separate clones. Both splice variants for introns 5 and 7 introduced a premature stop codon. Intron 1 of *Tcasα8* was spliced out 21 bases upstream (relative to previously reported sequences (Jones and Sattelle, 2007)) in all 10 clones I sequenced, but remained in-frame. The resulting 7 amino acid deletion resulted in a protein that was ~1% less similar to *α8* orthologs in *A. gambiae, A. mellifera,* and *D. melanogaster* (actually *Dβ2*) respectively. *Tcasα10* intron 5 has a splice variant that is 13 bp downstream of the normal boundary and introduces a premature stop codon. Splice variants from all subunits are preceded by the typical 3′–AG intron boundary as deduced from genomic DNA sequences. The only exception to the boundary rule is seen in *Tcasα2* where intron 3 is spliced out 5 bp downstream of the typical 3′–AG intron boundary. The splice variant is preceded by a –TG boundary when compared to other cDNA and genomic sequences.

4.3.4 Unspliced Introns

Tcasα3, α4, α9, α10, α11 and *β1* have transcripts which contain introns. These transcripts are not a result of genomic DNA contamination as they do not possess any other introns. Introns that were unspliced all had the typical GT-AG intron boundaries, introduced a premature stop codon, and were of short length (44-70 bp). A summary of effects these unspliced introns have on the mature transcripts is shown in Table 4.5. The unspliced introns do not have any polymorphisms associated with the intron or the adjacent exon sequences that could be associated with the inefficient splicing. Out of the subunits that exhibit unspliced introns, *Tcasα4* was the only subunit that had alternative exon usage. Therefore, it is unlikely that alternative exon usage is an important factor for retaining introns. Intron location also does not appear to be

Table 4.5 Length and intron number of unspliced introns from nAChR subunits in *T. castaneum*. All unspliced introns introduce premature stop codons.

Subunit	Unspliced Intron Length (bp)	Intron #	Detection Rate
Tcasα3	44 + 45	9 + 10	1/11
Tcasα4	45	7	1/15
Tcasα9	49 + 44	5 + 6	1/11
Tcasα10	50 + 44	2 + 3	1/12
Tcasα11	70	6	2/10
Tcasβ1	45	6	2/18

important for retaining introns, since they can appear early or late in the transcript. The only consistent variable in retaining introns in *T. castaneum* is short intron length (Table 4.5). Intron 9 was unspliced in both *Tcas*α*3* and *Amel*α*3* (Jones et al., 2006). While intron 9 in *Tcas*α*3* and *Amel*α*3* vary in length and 3′ splice site, the fact that the same intron is unspliced in two different taxa suggests a possible biological role for these truncated proteins. Although a premature stop codon is introduced, unspliced intron 9 in both *Amel*α*3* and *Tcas*α*3* would still produce a transcript coding for a protein with signal peptides, extracellular binding domains, and three transmembrane domains. Retention of introns 5 and 6 of *Tcas*α*9* disrupts the reading frame immediately before TM2 and introduces a premature stop codon. A *Tcas*α*10* transcript containing introns 2 + 3 codes for a protein containing only the signal peptide and loop D. The transcript of *Tcas*α*11*, which contains intron 6, is immediately truncated just after TM3. Retention of intron 6 in *Tcas*β*1* truncates the protein immediately before TM2. Intron 6 is immediately downstream and adjacent to the codons for the conserved GEK motif, which is integral for selective ion permeability (Jensen et al., 2005). Retained introns did not encode motifs such as N-glycosylation or phosphorylation sites or novel transmembrane domains.

4.3.5 RNA Editing

A-to-I RNA editing was found only for *Tcas*α*6*, and was similar to a previous report (Jones and Sattelle, 2007). All editing sites for *Tcas*α*6* are on exon 5. Editing sites 4, 5, and 6 (numbering based on all possible A-to-I RNA editing sites in insects (Jin et al., 2007)) were edited at a frequency of 86%, 51% and 55%, respectively, based on *Tcas*α*6* transcripts containing exon 5 (n = 29). Transcripts containing edits only at sites 4, 5, or 6, or combinations of 4+5, 4+6, 5+6, 4+5+6 were 14%, 0%, 7%, 21%, 17%, 0% and 31%, respectively. Editing at

site 5 was always in conjunction with editing at site 4. Previously reported (Jones and Sattelle, 2007) editing of *Tcasβ1* at nucleotide 155 (resulting in a K to R substitution) was not seen in any of the 18 clones I sequenced. Thus, the editing frequency of *Tcasβ1* is <6% in the GA-1 strain of *T. castaneum*.

4.3.6 Alleles

Alleles were observed in *Tcasα2*, *α3*, *α4*, *α5*, *α6*, *α7*, *α8*, *α9*, *α11*, and *β1*. Alleles were designated for substitutions that were not previously reported as A-to-I RNA editing sites and were shared between 2 or more independent clones or previously reported sequences. Only seven of 51 polymorphisms resulted in an amino acid substitution (Table 4.6). *Tcasα6*, *α8*, *α9*, and *α11* contained non-synonymous substitutions. While nucleotide transitions were more frequent, there was no statistical bias toward any specific nucleotide substitution across all alleles (one-way ANOVA, P> 0.05). Of the 51 polymorphic sites, 80% occurred at the third codon position, of which, only A1069G/T in *Tcasα9* resulted in an amino acid substitution.

4.4 Discussion

The sequences of nAChR subunit cDNAs that I report exhibit an unprecedented amount of variation in these genes from a single organism. While my work confirms a previous report (Jones and Sattelle, 2007), there are many novel differences in my data set. I observed a greater amount of variation in alternative exon usage in *Tcasα6* and identified 18 new transcripts. While more than 256 splice variants are mathematically possible from this arrangement of alternative exons, the 18 splice variants are likely to be an accurate measure of transcript diversity given the large number of clones I sequenced.

Table 4.6 Summary of *T. castaneum* nAChR subunit alleles. Only non-synonymous substitutions are listed.

Subunit	Base Subsitution[a]	Amino Acid Substitution	Detection Rate
α5	G268A	G90R	3/11
α6	T128C	V43A	2/39
α8	A314G	E105G	2/10
	T775C	C259R	2/10
α9	A1069G [v5]	T357A	1/11
	A1069T [v3]	T357S	1/11
α11	G36A	M12I	5/10

a = bases are numbered relative to the translation start site of *T. castaneum* subunits.
v3, v5 = allele specific polymorphisms. All alleles are v2 unless otherwise noted.

While almost all duplicated exons are spliced in a mutually exclusive manner (Letunic et al., 2002), I identified a transcript that contained both exons 3a and 3b (3ab). This pattern of alternative splicing of exon 3 in the α6 subunit was seen in *D. melanogaster* (Grauso et al., 2002), *M. domestica* (Gao et al., 2007c) and *B. mori* (Shao et al., 2007, Jin et al., 2007), but not previously described in *T. castaneum* (Jones and Sattelle, 2007). Splicing together exons 3a and 3b is developmentally regulated in *B. mori* (Jin et al., 2007). Having both exons 3a and 3b would duplicate the acetylcholine binding loop D. It is unknown if subunits with repeated ligand binding loops would alter ACh binding or not. Much like in *B. mori*, I found that exon 3 can be excluded from mature transcripts all together (Shao et al., 2007). Exclusion of exon 3 does not cause a frameshift in the transcript and otherwise retains nucleotide and amino acid identity to other transcripts. These transcripts would produce proteins that lack ligand binding loop D. Thus, the ligand binding capacity and affinity of these subunits would most likely be altered.

I observed alternative exons 8a and 8b in *Tcasα6*. The frequency of exon usage (Table 4.4) shows that 8b is found in transcripts more frequently than 8a. Exon 8c was not found in any clone that was sequenced, but a novel exon 8d was detected. The frequency of exon 8 usage I found in *Tcasα6* is 8a > 8b >> 8d, thus ensuring most *Tcasα6* transcripts would produce proteins with TM2 that contains amino acids crucial to normal receptor function. This is in agreement with previous reports from *T. castaneum, A. mellifera, B. mori*, and *D. melanogaster* (Jin et al., 2007) with the exception that exon 8c was found in the other insects instead of 8d. Although I did not find exon 8c, it has been shown that exon 8c is used infrequently in other insects (Jin et al., 2007, Gao et al., 2007c). There were 3 clones that did not contain exon 8. Exon 8 was also excluded from isoform IX in *M. domestica* (Gao et al., 2007c), but transcripts missing exon 8 may be non-functional as they lack the second transmembrane segment (TM2) and the well

conserved glutamic acid residue (encoded by transcripts containing exons 8a and 8b) that is involved in ion conductance (Imoto et al., 1988). Exclusion of exon 8 does not cause a frameshift in the sequence. These two physical alterations in the resulting protein may form complexes with altered pharmacological properties. Neither *Bmα5* from *B. mori* (Shao et al., 2007) nor *Agα1* from *Aphis gossypii* (Li and Han, 2005) posses transcripts that code for TM4 in any mature transcripts; therefore, it may be possible to generate smaller subunits lacking TMs that may retain function.

The previously undescribed exon 8d from *Tcasα6* appears to be an anomaly rather than a true alternative exon. The low nucleotide and amino acid similarity to 8a, 8b and 8c, longer exon length, longer intron length between exons, immediate introduction of a stop codon and low detection rate (1/39 clones) indicates exon 8d is either a splicing error or an optional exon rather than a true alternative exon that arose through duplication. This is similar to the observation of exon 9b in the *α7* subunit from mice (Saragoza et al., 2003). However, these two exons are not analogous as *Tcasα6* 8d truncates the protein at the beginning of TM2, while mouse *α7* terminates just after TM3.

The observation of optional exons 5 and 6+7 for *Tcasα6* is a rare event relative to other insect nAChRs. The only other examples of optional exon use are exon 2 of *Dα4* (Lansdell and Millar, 2000), exons 5-7 of *Mdα6* (Gao et al., 2007c) and exons 2 and 3 of *Agβ1* (Li and Han, 2005). Although *Tcasα6, Dα4* and *Mdα6* are alternatively spliced, *Agβ1* is not. Thus, alternative exon use is not a requirement for optional exon use.

Exon 5 of *Tcasα6* is an optional exon missing from 25% of the clones. Deletion of exon 5 introduces a premature stop codon. Exon 5 codes for extracellular loops E and B which are important for ligand binding. Given that exon 5 codes for amino acids crucial for receptor

function and diversity through ligand binding and RNA-editing, it is unclear why it would be excluded from 9 of the isoforms. However, mutual exclusion of exon 4 and exon 5, as in the case of isoform XIX, does not disrupt the open reading frame of the transcript. Exclusion of exon 3 and exon 5, though, does introduce a premature stop codon. Exon 5 is always missing from transcripts that are also missing both exons 6 and 7. Exon 6 codes for extracellular loop F and exon 7 codes for loop C, which contains the α-subunit specific YxCC motif.

A. gambiae (Jones et al., 2005), *A. mellifera* (Jones et al., 2006), *D. melanogaster* (Grauso et al., 2002), and B. *mori* (Shao et al., 2007) *α6* orthologs have identical genomic architecture. Exons 10, 11, and 12 of these *α6* orthologs in other insects are fused into a single exon 10 in *T. castaneum*. This is an interesting observation because *T. castaneum* is placed phylogenetically above *A. mellifera* and below the other insects. Exon boundaries for other orthologous nAChR subunits do not share the same conserved genomic architecture as seen in α6 orthologs.

Exons 8 through 10 of *Tcasα5* were not seen in any of the 11 clones I sequenced. I conclude that reported exons 8 through 10 are probably very rare exons for four reasons. First, exons showing such a high similarity at the nucleotide level would likely indicate alternative exons as alternative exons 4 and 4′ of *Tcasα4* share 70% nt similarity while exons 3a/b and 8a/b/c of *Tcasα6* share 66% and 60-65% similarity, respectively. The discrepancy in the length of exons 8 and 9, however, is not indicative of alternative exons because all other alternative exons in *T. castaneum* are exactly the same length. Second, the similarity of the adjacent areas of both exons to exactly match nearby introns suggests this stretch of DNA may have undergone recent duplication. This is substantiated by the fact that sequence comparison of both exons 8 and 9 with the adjacent introns (i.e. intron7/exon8/intron8 vs. intron 8/exon9/intron9) share 85%

similarity. Third, the primers designed specifically for exon 8 annealed to exon 9 without generating a double banding pattern on the gel suggests that exon 8 is not transcribed. Fourth, transcripts containing exons 9 and 10 do not contain other exons that are found in mature transcripts which may indicate these exons are processed differently than transcripts that do not contain them.

Splice variants in the 3′ splice site of introns were found in 4 subunits. For *Tcasα3*, *Tcasα6*, and *Tcasα10*, the splice variants disrupted the open reading frame and introduced a premature stop codon. All clones that I sequenced for *Tcasα8* had "splice variants" compared to previously reported sequences. It is unlikely that this is a true splice variant, but rather it is actually the normal splicing boundary as it is seen in all clones. The bp length of this "splice variant" coded for an additional 7 amino acids in the highly variable N-terminal signal leader peptide and did not disrupt the open reading frame. Therefore, the differences between the deduced amino acid sequence previously reported (Jones and Sattelle, 2007) and mine would not be expected to have a dramatic effect on the normal function of the receptor complex, but might alter its cellular trafficking. It is difficult to reconcile the differences seen in *Tcasα8* compared to the previous report because the beetle strains used in my study and by Jones *et al.* (Jones and Sattelle, 2007) are highly related (i.e. GA-2 is derived from GA-1) and the techniques used in both studies are very similar. Although the "splice variants" I reported for *Tcasα8* decreased similarity to other orthologous subunits, it does not create significant changes in overall similarity (1.0 to 1.3%).

Unspliced introns were found in six nAChR subunit transcripts in *T. castaneum* and they all introduce premature stop codons. This represents the largest collection of truncated nAChR transcripts due to unspliced introns in any insect and may be a reflection of the large number of

clones I sequenced. Unspliced introns were also reported from *Dα7* (Grauso et al., 2002), *Agamα7* (Jones et al., 2005), *Amelα3* and *α7* (Jones et al., 2006), and *Mdα6* isoform VIII (Gao et al., 2007c). All unspliced introns introduce a premature stop codon shortly after the introduction of the intron into the open reading frame except in *Agamα7*, which remains in frame for an additional 86 amino acids. Intron 9 in *Mdα6* isoform VIII was only 59 bp in length. While I did not observe any retained introns in *Tcasα7*, intron 5 was unspliced in *Agamα7*, *Amelα7*, and *Dα7* with lengths of 7292, 3666, and 62 bp respectively. Thus, it appears intron length may be a species specific determinant to leave introns unspliced from mature transcripts. Unspliced intron 9 in *Tcasα3* and *Amelα3* and intron 6 of *Tcasα11* produce transcripts that are truncated just after TM3 while unspliced intron 7 of *Tcasα4* is truncated halfway through TM3. Similarly truncated transcripts of *Dα1* abolished ACh-mediated inward currents (Schulz et al., 2000). EMS mutagenesis that produced truncated *Dβ2* between TM3 and TM4 in *D. melanogaster* created a strain resistant to imidacloprid and other neonicotinoids (Perry et al., 2008). Thus, investigating the role of orthologous subunits of *T. castaneum* in insecticide toxicity would be of great value.

Alleles were found for 10 nAChR subunit genes and some of the non-synonymous substitutions could affect receptor function. In *Tcasα5*, the G90R substitution occurs at a highly conserved glycine residue between loops D and A. Glycine at position 90 (based on *Tcasα5* numbering) is highly conserved in *T. castaneum* (11/12 nAChRs). Alignments show it is also conserved in *Dα1-7*, *Dβ1* and *2*, *Hvα7-1* and *2* (*Heliothis virescens*), *Ggα7* (*Gallus gallus*) *Tcα1* (*Torpedo californica* (Grauso et al., 2002)), *Agamα1-8* and *β1* (Jones et al., 2005), *Amelα1-9* and *Amelβ1* (Jones et al., 2006), *Bmα1-8*, *Bmβ1-3* (Shao et al., 2007), *Cfα1-5,7,8* and *β1* (*Ctenocephalides felis* (Bass et al., 2006)) *Mdα6* (Gao et al., 2007c), *Mdα5* and *β3* (Gao et al.,

2007b), *Mpα1-5* (*Myzus persicae* (Huang et al., 1999)). The striking conservation within and across insect and vertebrate taxa suggests that G90 has an important role on subunit structure and function. The effect of the G90R substitution is not known. The addition of a charge at such a conserved site, however, is likely to cause some structural change. The V43A substitution in *Tcasα6* falls between the signal peptide and loop D. Substitution E105G in *Tcasα8* occurs two residues downstream of the well conserved glycine residue mentioned in *Tcasα5*. This residue can be acidic or basic so replacing glutamic acid with glycine at this position may affect proper folding of the extracellular loops. A fairly conserved cysteine (9 of 12 subunits in *T. castaneum*) at position 259 found in TM1 is changed to arginine in *Tcasα8*. Position 1069 in *Tcasα9* is the only allele that has a double variant (A1069G/T). Both of these substitutions result in T357A/S at the amino acid level. This is located adjacent to a potential phosphorylation site (SFH). The T357A/S substitutions occur at the intracellular linker between TM3 and TM4 which is important for cell surface expression (Ren et al., 2005). The M12I substitution resulting from the G36A allelic variant of *Tcasα11* occurs in the highly variable signal peptide. The codon location of the alleles is biased at the third position. Since only 1/35 substitutions at the third codon position create an amino acid substitution, they are likely to be under little selective pressure and thus able to accumulate at this position. Nearly 80% of *T. castaneum* nAChR alleles are substitutions at the third codon position.

nAChR subunits in the GA-1 strain of *T. castaneum* undergo extensive post-transcriptional modification and some contain multiple alleles. Comparison of results between the GA-1 (presented here) and GA-2 (Jones and Sattelle, 2007) strains shows that although strains may be highly related, there may be dramatic differences between RNA processing and allelic content. Future research on nAChRs is needed to shed light on the functional role of

transcripts with alternative exons, retained introns and splice variants. The easy manipulation *T. castaneum* using RNAi, chemical mutagenesis, and binary transposon systems (Consortium, 2008) presents an excellent opportunity to study these interactions.

4.5 Acknowledgements

I thank Kathy Leonard for providing the GA-1 strain of *T. castaneum*. This work was supported by a Sakaria Fellowship.

Chapter 5

Limitations of RNAi of α6 nicotinic acetylcholine receptor subunits on assessment of the target site of spinosad

5.1 Introduction

The nAChRs mediate excitatory cholinergic neurotransmission in the central nervous system of insects. The nAChRs are members of the Cys-loop ligand-gated ion channel superfamily which includes receptors for serotonin, glycine and GABA (Ortells and Lunt, 1995). The nAChR contains three functional domains: an extracellular N-terminal ligand-binding domain, four transmembrane segments (TM1-4), of which TM2 forms the pore of the receptor channel, and the intracellular linker between TM3-4, which is responsible for receptor desensitization and intracellular trafficking (Corringer et al., 1995, Imoto et al., 1988, Millar and Harkness, 2008). Native nAChRs of insects are homopentamers of α subunits, or heteropentamers of α and β subunits (Sattelle et al., 2005, Millar, 2009, Millar and Denholm, 2007). The α subunits possess a YxCC motif in loop C of the ligand-binding domain, whereas β subunits do not. Insect have between 10 and 16 nAChR genes (Sattelle et al., 2005, Jones and Sattelle, 2007, Shao et al., 2007, Jones et al., 2010). Insects can generate tremendous diversity of nAChR subunit proteins through post-transcriptional modifications such as alternative splicing and A-to-I RNA editing. For example, alternative and cassette exon use in *Tcasα6* from *Tribolium castaneum* can generate more than 256 splicing isoforms (Rinkevich and Scott, 2009), and more than 30,000 unique transcripts are possible from *Dα6* of *Drosophila melanogaster* using combinations of alternative splicing and A-to-I RNA editing (Grauso et al., 2002). The post-transcriptional modifications of insect α6 subunit orthologs are evolutionarily conserved

(Tian et al., 2008, Jin et al., 2007). The nAChRs are the target for two major groups of insecticides: spinosyns, such as spinosad, and neonicotinoids, such as imidacloprid. However, the role of individual nAChRs (and their variously spliced and edited forms) in modulating responses to insecticides are not well known.

Macrocyclic lactones, such as spinosad, are a widely used and economically important class of insecticides. Spinosad has been used to successfully control many agricultural, domestic and public health pests (Salgado and Sparks, 2005, Stough et al., 2009). This wide spectrum of effectiveness, combined with reduced impacts on non-target insects and the environment and low vertebrate toxicity are all desirable and beneficial aspects of spinosad (Salgado and Sparks, 2005, Thompson et al., 2000). Spinosad exerts its toxic effects through interactions with the nicotinic acetylcholine receptor by acting as an allosteric modulator (Salgado and Sparks, 2005). Through a series of mutagenesis experiments it has been shown that *D. melanogaster* strains lacking Dα6 are 370-1100 fold resistant to spinosad (Perry et al., 2007, Watson et al., 2010). In addition, spinosad resistance in diamondback moth is due to errant splicing, that introduces premature stop codons in *Pxylα6* (Rinkevich et al., 2010). These results clearly indicate a role for *α6* subunits in the mode of action of spinosad.

RNAi has been used in numerous biological fields, including insecticide toxicology (Rajagopal et al., 2002, Lycett et al., 2006, Bautista et al., 2009). However, the use of RNAi to test the role of different nAChRs in insecticide mode of action has not been reported. If RNAi could be used to effectively silence one or more nAChR subunits, this would allow for new studies that could elucidate the relative importance of each receptor to insecticide toxicology. As a starting point, we used RNAi of *α6* in two species of insect (*D. melanogaster* and *T. castaneum*) to examine the effect on toxicity to spinosad, given that α6 null strains are 370-1100

fold resistant to spinosad. While the levels of *α6* transcripts could be reduced using Gal4-driven expression of dsRNA (*D. melanogaster*) or dsRNA injection (*T. castaneum*), the toxicity to spinosad did not change. Possible reasons for these unexpected results are discussed.

5.2 Materials and Methods

5.2.1 Insects

Six strains of *Drosophila melanogaster* were used in this study. The actin and elav strains expressed Gal4 ubiquitously and in the nervous system, respectively, and were balanced over CyO. The Dα6RNAi strain expressed dsRNA under the control of UAS. The Df(2L)s1402 and Df(2L)Exel6025 strains had chromosomal deletions that lacked the Dα6 locus. The w^{1118} strain is a partial deletion of the *white* gene. The actin (stock# 25374), Dα6RNAi (#25835), Df(2L)s1402 (#556), Df(2L)Exel6025 (#7508), and w^{1118} (#3605) strains were obtained from the Bloomington Stock Center. The elav strain was kindly provided by Hanna Kim (Cornell University, Dept. Neurobiology and Behavior).

Reciprocal crosses of flies expressing Gal4 under specific promoters and Dα6RNAi were performed to activate the expression of the dsRNA (i.e., actin♀ x Dα6RNAi♂ and Dα6RNAi♀ x actin♂). The offspring that had the CyO marker were discarded so that only flies expressing dsRNA for *Dα6* were used in bioassays. Flies were reared on cornmeal-agar based media supplemented with dextrose and held at 25°C, 30% RH, and 12:12 light-dark photoperiod. Flies were transferred to new vials weekly.

One strain of red flour beetles (*Tribolium castaneum*) was used in this study. The wild-type, insecticide-susceptible GA-1 strain obtained from Kathy Leonard at Kansas State University and reared as previously described (Rinkevich and Scott, 2009).

5.2.2 RNA Extraction, cDNA Synthesis and qPCR of *Dα6*

Total RNA was isolated from 5, unsexed, 1-5 day old *D. melanogaster* adults using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Total RNA was resuspended in 100 μl of DEPC treated H_2O. RNA concentration was measured on a NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE). Reverse transcription was performed with 5 μg of total RNA using Go-Script (Promega, Madison WI) according to the manufacturer's directions.

Expression of *Dα6* relative to *actin5c* was evaluated in triplicate for each sample using 20 μl Power SYBER (Applied Biosystems, Carlsbad CA) with 25 ng of cDNA on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules CA). The primer set RTActinF and RTActinR was used to measure *actin5c* expression and the primer set RTDa6F and RTDa6R was used to measure *Dα6* expression (Table 5.1). Three independent batches of cDNA from each cross and parental strain were used. The thermocycler program was as follows: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 58^{o}C for 1 min. Fluorescence was measured at the end of each cycle. Relative expression of *Dα6* was evaluated using the $2^{-\Delta\Delta Ct}$ method and statistically compared using Student's T-test.

5.2.3 RNA Extraction, cDNA Synthesis and *In Vitro* Production of *Tcasα6* dsRNA

RNA isolation and cDNA synthesis were performed on 5, unsexed, 1-5 day old *D. melanogaster* adults as described above. A 723-bp fragment of *Tcasα6*, corresponding to base 1025 of the open reading frame through base 251 of the 3′ untranslated region of the mRNA, was amplified by PCR in 100 μl Go-Taq (Promega, Madison WI) reactions using the primers

Table 5.1 Sequences of primers used**.**

Primer	**Sequence**
T7Tcasa6F0	TAATACGACTCACTATAGGGTCTTACAATGGCTGCCGTGGATG
T7Tcasa6R1	TAATACGACTCACTATAGGGTCGCAAATTCGCAAAATCGCAGA
Tcasa6F1	CACGAAAAGCGGCTACTAAACA
Tcasa6R1	GTTTGTTTGGAAAGTCCCGTCGA
RTActinF	CCCAAGGCCAACCGTGAGAAGATG
RTActinR	GACCGGAGGCGTACAGCGAGAGC
RTDa6F	CGTCGCTGTCGCTGTTTGTCC
RTDa6R	CAGGGGCTCCGATTCATTGG

T7Tcasa6F0 and T7Tcasa6R1 (Table 3.1). The PCR conditions were as follows: 94^{o}C for 2 min, followed by 35 cycles of 94^{o}C for 30 sec, 60^{o}C for 30 sec, and 72^{o}C for 1.5 min with a final extension step at 72^{o}C for 10 min. The PCR product was purified by QIAEXII gel purification kit (Qiagen, Valencia, CA) after electrophoresis on 1% agarose gel. The purified product was used as DNA template for dsRNA synthesis.

Synthesis of dsRNA was performed using the AmpliScribe T7-Flash transcription kit (Epicentre, Madison WI) according to the manufacturer's instructions. The transcribed product was DNase I treated, then phenol/chloroform (1:1) and chloroform extracted, respectively, and precipitated with ammonium acetate, followed by a 70% ethanol wash. The dsRNA pellet was dissolved in distilled H_2O at a concentration of 3μg/μl and stored at -70°C until use.

One-day old *T. castaneum* pupae were dorsally fixed to double-sided tape on a glass slide and injected with 600 ng of dsRNA for *Tcasα6* (in a 0.22 μl volume) between the second and third ventral abdominal segments of using a Picospritzer II pressure system (General Valve Corp, Fairfield, NJ). The same amount of distilled H_2O was injected for control. Uninjected beetles were used as an injection control. The injected pupae were placed into wheat flour fortified with 5% (wt/wt) yeast after one hour incubation at room temperature and kept in continuous darkness at 27°C and about 65% RH for one week until they emerged as adults to be used in bioassays.

RNA extraction was performed as described above on control injected and *Tcasα6* dsRNA injected beetles one week after injection. RT-PCR for *Tcasα6* was conducted using 1 μg of RNA in 25 μl One-Step RT-PCR reactions (Qiagen, Valencia CA) according to the manufacturer's directions with the primers Tcasa6F1 and Tcasa6R1 (Table 5.1). The PCR conditions were as follows: 50^{o}C for 60min, 94^{o}C for 2 min, followed by 25 cycles of 94^{o}C for

30 sec, 65°C for 30 sec, and 72°C for 30 sec with a final extension step at 72°C for 10 min. The PCR was visualized on a 1% agarose gel.

5.2.4 Spinosad Bioassays

Spinosad (96%, Elanco Animal Health, Indianapolis, IN) was dissolved in acetone to create 20 mg/ml stock solutions from which the appropriate serial dilutions were made. Contact bioassays were performed by treating a scintillation vial (Wheaton, Millville, NJ; internal surface area = 38.6 cm^2) with 0.5 ml of insecticide solution. Controls vials were treated with 0.5 ml of acetone only**.** Vials were placed in a fume hood and rolled to evenly coat the inner surface of the vials. Treated vials were allowed to dry for 1 hr. Twenty, unsexed, 1-5 day old adult flies or beetles were placed in a vial covered with white nylon tulle and plugged with a cotton ball. The cotton balls were wetted daily with 10% sucrose water. Bioassays were conducted at the rearing conditions used for each insect as described above. A minimum of three replicates for each concentration that gave more than 0% and less than 100% mortality were performed. Mortality was assessed at 72 hrs post treatment.

Bioassay data were pooled for each replicate and analyzed by standard Probit analysis as adapted for personal computer use (Raymond, 1985) using Abbott's correction for control mortality (Abbott, 1925). The LC_{50} values of each strain, cross (flies) and dsRNA treatment (beetles) were considered to be significantly different if the 95% confidence intervals did not overlap.

5.3 Results

5.3.1 Flies

The Gal4-UAS system significantly reduced the expression of *Dα6* to 25-44% relative to the parental Dα6RNAi strain using actin and elav crosses (Student's T-test, $p<0.05$ (Table 5.2)).The spinosad LC_{50} values of each cross were significantly higher than the parental driver strains (2-5 fold), but there were no significant differences in the LC_{50} values compared to the Dα6RNAi parental strain (Table 5.2). Bioassays with the Dα6 deficient strains Df(2L)s1402 and Df(2L)Exel6025 showed very high levels of spinosad resistance (1244 and 7408-fold, respectively) compared to the w^{1118} strain from which they were derived (Table 5.2), which agrees with previous work (Perry et al., 2007, Watson et al., 2010).Therefore, although *Dα6* null strains are highly resistant to spinosad, reduced expression of *Dα6* by RNAi is insufficient to significantly alter spinosad sensitivity.

5.3.2 Beetles

Based on semi-quantitative RT-PCR, *Tcasα6* dsRNA injection reduced *Tcasα6* expression by nearly half (Figure 5.1). Nevertheless, there were no significant differences in the spinosad LC_{50} values between control (LC_{50}=618 ng/cm^2 (95% CI 446-891)), sham injected (LC_{50}=130 ng/cm^2 (95% CI 85-497)), and *Tcasα6* dsRNA injected beetles (LC_{50}=444 ng/cm^2 (95% CI 203-1056)). These results mirror the lack of change in spinosad insensitivity despite reduction in *α6* expression as seen in *D. melanogaster* (Table 5.2).

Table 5.2 Spinosad bioassay results and *Dα6* expression from strains and crosses of *D. melanogaster*.

Strain or Cross	n†	Spinosad LC_{50} (ng/cm^2)‡	Slope§	Rel. *Dα6* Expression!
w^{1118}	560	4.9 (3.9-6.0)	1.9 (± 0.2)	NA
Df(2L)s1402	630	6100 (2190-9270)	0.9 (± 0.1)	NA
Df(2L)Exel6025	646	36300 (22000-63500)	0.6 (± 0.1)	NA
actin	1100	25.0 (22.4-28.0)	2.4 (± 0.2)	NA
elav	672	15.1 (12.0-18.5)	1.3 (± 0.1)	NA
Dα6RNAi	431	63.1 (52.0-79.6)	2.4 (± 0.2)	1.00[a] (0.07)
actin x Dα6RNAi	262	57.9 (31.5-90.7)	1.2 (± 0.2)	0.44[b] (0.05)
Dα6RNAi x actin	250	84.1 (39.0-146.0)	0.9 (± 0.1)	0.40[b] (0.04)
elav x Dα6RNAi	385	75.5 (60.5-87.0)	3.2 (± 0.6)	0.44[b] (0.16)
Dα6RNAi x elav	930	74.6 (64.6-83.3)	2.7 (± 0.3)	0.25[b] (0.02)

Different letters next to values in columns represent significantly different values.
† = number of animals tested
‡ = numbers in parenthesis represent the 95% confidence interval of the LC_{50}
§ = number in parenthesis is the standard error
! = NA indicates not assessed, number in parenthesis is the standard deviation

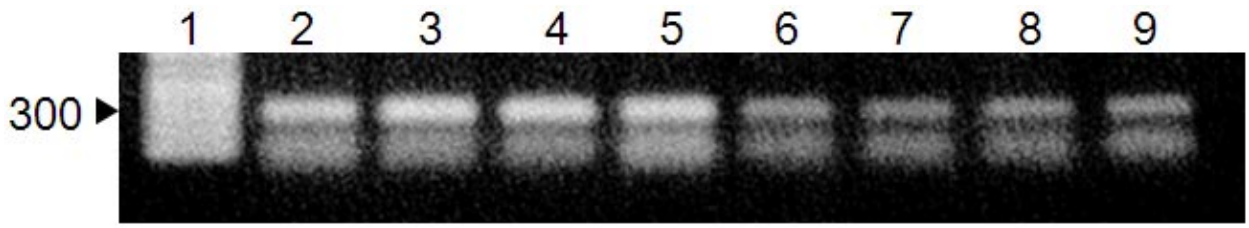

Figure 5.1 Semiquantitative PCR analysis of *Tcasα6* expression in *T. castaneum*. Lane 1 is 5 µL of 1-kb Plus Ladder (Invitrogen, Carlsbad CA), lanes 2-5 are bands from control injected beetles, and lanes 6-9 are from beetles injected with *Tcasα6* dsRNA. The number to the left of lane 1 indicates the size of the band in lane 1 in bp. This gel demonstrates a nearly 50% reduction in *Tcasα6* expression via *Tcasα6* dsRNA injection. Gel image courtesy of Dr. J.-R. Gao.

5.4 Discussion

Based on those results, it appears that RNAi of nAChRs has limited utility for studies of spinosad and perhaps other insecticides working at this target site. There are a few reasons why this may be the case: recessive pattern of inheritance, protein stability, difficulties in dsRNA uptake in the nervous system, or intrinsic properties of the species, gene, or tissue.

Spinosad resistance in *α6* null strains is highly recessive (Perry et al., 2007, Rinkevich et al., 2010), and insecticides may only need to affect a small number of target sites to exert their toxic effects (Tatebayashi and Narahashi, 1994). Thus, the inability of RNAi to completely abolish expression of *Dα6* may explain the ineffectiveness of RNAi on the LC_{50} of spinosad in my experiments.

It is possible that the rate of protein turnover for nAChRs is an important factor for the effectiveness of RNAi, and nAChRs can be stable for more than 2 weeks (Lomazzo et al., 2011). Whether or not nAChR protein stability contributed to our seeing no change in spinosad susceptibility is difficult to determine because transcripts were still detected in strains subjected to our RNAi protocols.

There may be difficulties with dsRNA uptake and RNAi effectiveness in specific tissues. For example, larval injection of dsRNA to assess RNAi in the salivary glands of *Anopheles gambiae* requires 10-fold higher amounts of dsRNA to reduce gene expression to similar levels in the midgut and ovary (Boisson et al., 2006). However, in our experiments, the 600 ng of *Tcasα6* dsRNA injected should have been adequate to induce RNAi and effectively reduce expression of *Tcasα6* because minute amounts dsRNA can cause a shift in phenotype in *T. castaneum* larvae (Tomoyasu and Denell, 2004).

The effectiveness of RNAi may be intrinsic of the species, gene, or tissue (Belles, 2010). RNAi has been effectively used in *D. melanogaster* and *T. castaneum*, so species specific factors such as dsRNA degradation efficiency, spread of RNAi signaling, and muted response of RNAi machinery are not a cause of the ineffectiveness of RNAi in our experiments. Properties of the gene may be involved with the ineffectiveness of RNAi for the *α6* subunit, but these prospects are speculative at best. Gene specific features of RNAi insensitivity include *α6* dsRNA degradation, increased transcription rate of *α6* subunits upon dsRNA administration, and protection from RNases. The likely cause of the ineffectiveness of RNAi for *α6* subunits is dependent on tissue specific factors such as the observation that RNAi machinery is not highly expressed in the nervous system. In *D. melanogaster*, the mRNA abundance for both *Dicer1* and *Dicer2* in the adult brain and thoracicoabdominal ganglion is much lower than *Dα6* expression in those tissues (Figure 5.2 (Chintapalli et al., 2007)). The disparity in expression of these genes in these tissues may explain the ineffectiveness of *Dα6* RNAi in evaluating spinosad sensitivity. Another study has shown that RNAi of nAChRs is more difficult than other genes. In the brown planthopper, *Nilaparvata lugens*, injection of dsRNA for globally expressed calreticulin and the gut specific cathepsin-B decreased expression of those genes by 40%, but injection of dsRNA for *Nlβ2*, a nAChR subunit, only reduced expression by 25% (Liu et al., 2010). The effect of RNAi for *Nlβ2* on insecticide sensitivity has not been evaluated.

Difficulties of tissue penetration in the nervous system is likely not the cause of ineffective RNAi, because RNAi of *dAdar*, which is responsible for A-to-I RNA editing in the nervous system in *D. melanogaster*, is very effective and informative (Jepson and Reenan, 2009). The expression levels *dAdar*, *Dicer1* and *Dicer2* in the brain and thoracicoabdominal ganglion are similar (Figure 3.2 (Chintapalli et al., 2007)), thus supporting the hypothesis that

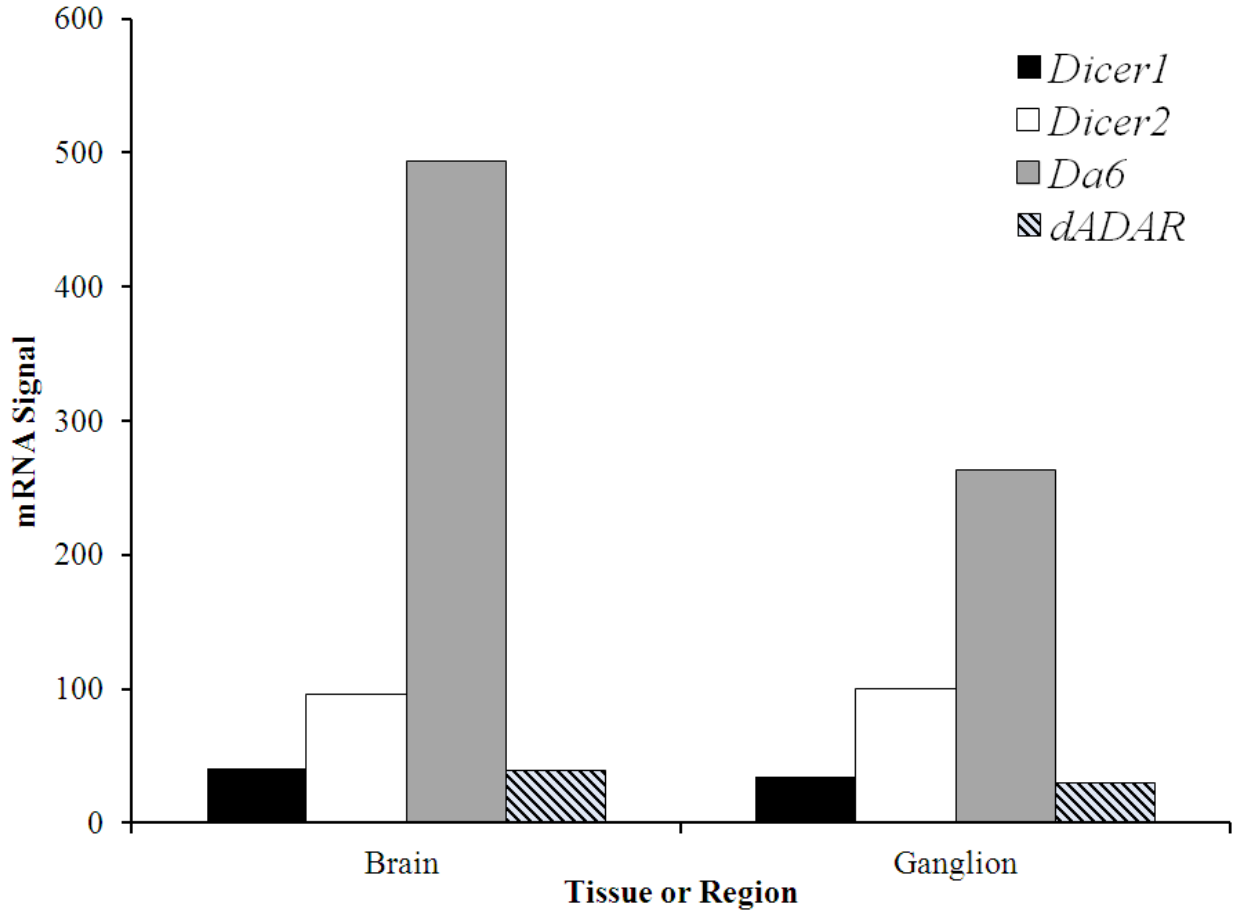

Figure 5.2 Expression levels of *Dicer1*, *Dicer2*, *Dα6*, and *dADAR* in the brain and thoracicoabdominal ganglion in *D. melanogaster*. Data from FlyAtlas (Chintapalli et al., 2007).

large differences in expression levels of RNAi target genes and *Dicer1* and *Dicer2* may be responsible for the incomplete suppression of *α6* in our experiments.

Based on the above, it appears that the most likely explanations for why spinosad resistance was not observed in our *α6* RNAi experiments was that complete suppression of the *α6* subunit may be required to generate spinosad resistance, but only 60-75% reduction in *α6* transcripts was achieved using RNAi. It appears the most likely explanation for the incomplete suppression of *α6,* particularly in *D. melanogaster*, was due to the difference in the expression levels of *Dα6* (high) and *Dicer1* and *Dicer2* (low). This hypothesis is supported by other reports that demonstrate the lack of complete silencing of other nAChR subunits (Liu et al., 2010, Vermehren and Trimmer, 2005).

Our bioassays reinforce the potential problem of evaluating the effectiveness of RNAi using a single concentration or dose of insecticide (Revuelta et al., 2009, Rajagopal et al., 2002, Lycett et al., 2006, Revuelta et al., 2011, Zhang et al., 2010). Results from using a single concentration may be misleading because they assume the slopes of the concentration response curves between test populations are equal. A one-concentration survivorship test would result in Type 1 error because the slopes varied between treatments (Table 5.2). For example, the LC_{50} values for Dα6RNAi, actin x Dα6RNAi and Dα6RNAi x actin were not significantly different (Table 5.2). However, if we used the LC_{95} of spinosad for the Dα6RNAi strain, the actin x Dα6RNAi and Dα6RNAi x actin would have only shown 75% and 66% mortality, respectively, giving the impression that ubiquitous reduction in *Dα6* expression decreases spinosad sensitivity (Figure 5.3). Conversely, if the reciprocal crosses of actin and Dα6RNAi were tested at the LC_5 of spinosad to the parental Dα6RNAi, the higher mortality of the cross offspring (~24%) would suggest that ubiquitous reduction of *Dα6* increases spinosad sensitivity (Figure 5.3). This

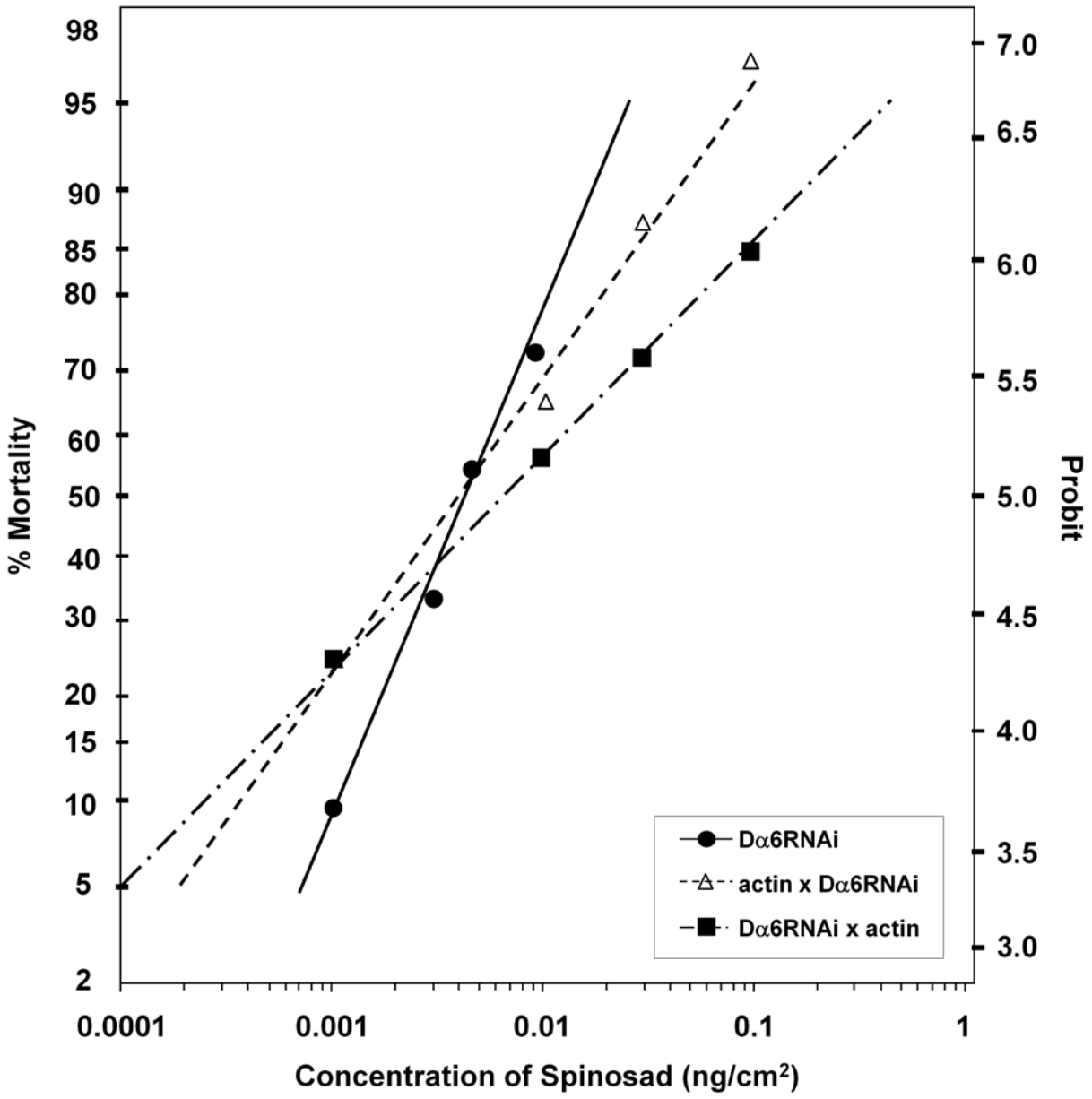

Figure 5.3 Log concentration-probit graph of spinosad toxicity to three lines of *D. melanogaster*. Concentrations that gave 0 or 100% mortality are not shown.

demonstrates that the dose or concentration applied to test for insecticide sensitivity may yield contradictory and inaccurate assessments of the effect of RNAi on insecticide sensitivity, and all future experiments in this field should be considerate of this fundamental aspect of toxicology.

RNAi has been effectively used to study critical processes such as embryogenesis, differentiation, digestion, and sex-determination in diverse insects such as *Drosophila*, *Tribolium*, and *Bombyx* (Miller et al., 2008, Parthasarathy and Palli, 2009, Hossain et al., 2008). RNAi in *Tribolium* may require as little as 5 pg of dsRNA to produced a noticeable phenotypic change and the reduced systemic expression of genes via injection of dsRNA can last up to two months (Tomoyasu and Denell, 2004). Recent developments have explored the use of RNAi as a form of agricultural pest control by administering dsRNA as a spray or in transgenic plants (Huvenne and Smagghe, 2010). While these applications of RNAi have yielded valuable insights to the function of many genes, we were not able to produce significant phenotypic changes in spinosad sensitivity by using RNAi for the same target using two methods in two different insects. My results suggest RNAi against nAChRs may not be an appppropriate method to study the role of individual nAChRs in insecticide toxicology.

5.5 Acknowledgements

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Chapter 6

A-to-I RNA Editing Affects the Sensitivity of Spinosad and Imidacloprid to *Drosophila melanogaster*

6.1 Introduction

Spinosyns such as spinosad, and neonicotinoids such as imidacloprid, are two widely used classes of insecticides (Jeschke et al., 2011, Salgado and Sparks, 2005). Spinosad and imidacloprid both exert their toxic effects through interactions with the nicotinic acetylcholine receptor, although their exact mechanisms of action on nAChRs are distinct (Salgado and Sparks, 2005, Jeschke and Nauen, 2008).

Nicotinic acetylcholine receptors (nAChRs) are members of the cys-loop ligand-gated ion channel superfamily that includes serotonin, glycine and GABA receptors (Ortells and Lunt, 1995). Ligand binding occurs at the extracellular N-terminal domain (Corringer et al., 1995). Ion currents through the receptor are mediated by the second of four transmembrane domains (Imoto et al., 1988). The intracellular linker between the third and fourth transmembrane domains is responsible for receptor desensitization and intracellular trafficking (Millar and Harkness, 2008). In insects, nAChRs are found on post synaptic sites of neurons in the central nervous system and native nAChRs of insects are homo or heteropentamers of α and β subunits, although the exact composition is largely unresolved (Sattelle et al., 2005, Millar, 2009, Millar and Denholm, 2007). Insects have between 10 and 16 nAChR genes (Sattelle et al., 2005, Jones and Sattelle, 2007, Shao et al., 2007, Jones et al., 2010). Evolutionarily conserved patterns of post-transcriptional modifications such as alternative splicing and A-to-I RNA editing create a diverse array of transcripts (Rinkevich and Scott, 2009, Tian et al., 2008, Jin et al., 2007, Grauso et al., 2002).

Insecticide resistance to spinosad or imidacloprid can arise though gene deletions, altered post-transcriptional modifications, or single nucleotide polymorphisms in nAChR subunits. Null mutations in *Dα6* of *Drosophila melanogaster* are 370-1100 fold resistant to spinosad (Perry et al., 2007, Watson et al., 2010). The highly spinosad resistant Pearl-Sel strain of the diamondback moth, *Plutella xylostella*, produces no full length transcripts of *Pxylα6*, and this trait is genetically linked with resistance (Rinkevich et al., 2010). Neonicotinoid resistance due to changes in nAChR subunits has been thoroughly investigated in the brown planthopper, *Nilaparvata lugens*. A single Y151S mutation in *Nlα1* of the R-T35 strain was associated with imidacloprid resistance (Liu et al., 2005). Receptors containing the $Nl\alpha1^{Y151S}$ mutation were less sensitive to imidacloprid (Liu et al., 2005, Liu et al., 2006). An A-to-I RNA editing site of *Nlβ1* was edited at a significantly higher rate in an imidacloprid resistant strain of *N. lugens* compared to a susceptible strain and receptors with this edited site were less sensitive to imidacloprid (Yao et al., 2009). These results indicate that the α6, and the α1 and β1 nAChR subunits are targets of spinosad and imidacloprid, respectively. These subunits undergo A-to-I RNA editing (Yao et al., 2009, Grauso et al., 2002, Hoopengardner et al., 2003), but the contribution of A-to-I RNA to *in vivo* sensitivity to spinosad and imidacloprid has not been evaluated.

A-to-I RNA editing is catalyzed by the enzyme Adenine Deaminase Acting on RNA (ADAR), which converts adenosine to inosine in mRNA. Inosine is recognized by guanosine by the translational and splicing machinery of the cell. This genetic recoding may cause amino acid substitutions, splice site variations or modify the levels of transcripts (Bass, 2002).

A-to-I RNA editing can affect behavior, neuroanatomy, and function of neuronal receptors and channels of insects. In *D. melanogaster*, *dAdar* null mutants have a disorganized nervous system morphology and deficits in motor control that grow progressively worse with age

(Palladino et al., 2000b). Flies lacking *dAdar* did not fly or exhibit diurnal activity patterns and displayed temperature sensitive paralysis (Jepson and Reenan, 2009). Additionally, *dAdar* deficient male flies take longer to initiate courtship behaviors and have an altered courtship song waveform (Jepson et al., 2011). Editing causes an R122G substitution in the extracellular ligand binding domain of the *Rdl* subunit of GABA-gated chloride receptors of *D. melanogaster*. GABA sensitivity and maximum current responses were significantly reduced in receptors with the R122G substitution (Jones et al., 2009). In the cockroach *Blatella germanica,* the voltage-dependence of activation is hyperpolarized by 7 mV in sodium channels that are edited to introduce an R184K substitution (Song et al., 2004).

The goal of my research was to evaluate the effect of A-to-I RNA editing on the sensitivity of nAChRs to both spinosad and imidacloprid. I used the Gal4-UAS system to drive expression of dsRNA for *dAdar* in different tissues of *D. melanogaster* to evaluate the role of A-to-I RNA editing on spinosad and imidacloprid toxicity. Spinosad insensitivity varied due to the tissue specific reduction of *dAdar*. Imidacloprid insensitivity increased when *dAdar* expression was reduced in cholinergic neurons, glia and muscle. These results indicate that while A-to-I RNA editing may affect traditional insecticide targets (i.e. nAChRs), previously unknown factors that are subject to A-to-I RNA editing may also be important for insecticide interactions.

6.2 Materials and Methods

6.2.1 *Drosophila* Strains

Seven strains of *Drosophila melanogaster* were used in this study (Table 6.1). Reciprocal crosses of flies expressing Gal4 under specific promoters and dAdar were performed to activate the expression of the dsRNA. In all crosses, the parental female is indicated by the strain first

Table 6.1 List of *D. melanogaster* strains used in this experiment.

Strain	**Bloomington Stock #**	**Note**
actin	25374	Gal4 expressed ubiquitously
elav	A	Gal4 expressed in nervous system
elav3	8760	Gal4 expressed in nervous system
cha	A	Gal4 expressed in cholinergic neurons
dj667	8171	Gal4 expressed in adult muscle
repo	7415	Gal4 expressed in glia
dAdar	28311	dsRNA for *dAdar* under UAS control

A= Obtained from Hanna Kim, Cornell University

appearing in the cross description (i.e. actin♀ x dAdar♂). Flies were reared on cornmeal-agar based media supplemented with dextrose and held at 25°C, ±30% RH, and 12:12 Light-Dark photoperiod. Flies were transferred to new vials weekly.

6.2.2 RNA Isolation and Reverse Transcription

Total RNA was isolated from 10 flies (5♀ and 5♂) from each strain and cross using TRIzol (Invitrogen, Carlsbad CA) according to the manufacturer's directions. The RNA was dissolved in 50 µl of DEPC-treated water. Reverse transcription was performed with 5 µg of total RNA using Go-Script (Promega, Madison WI) according to the manufacturer's directions.

6.2.3 PCR and Estimation of RNA Editing

An 854 bp fragment corresponding to nt 93-947 of the open reading frame of *Dα6* was amplified with the primers Dα6ORF-F3 and Dα6ORF-R3 (Table 6.2). A control reaction that amplified a 496 bp portion of the *actin5C* open reading frame from nt 465-961 was performed using the primers DmelActinF and DmelActinR (Table 6.2). Reactions were performed in 100 µl Go-Taq reactions (Promega, Madison WI) under the following thermocycler conditions: 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. PCR products were purified with the Wizard PCR Purification kit (Promega, Madison WI) and sent for sequencing with the Dα6InternalR2 primer at Cornell's Biotechnology Resource Center. Electropherograms were visualized in Chromas (Technelysium, Brisbane AUS). The height of the peak at each editing site was measured using Photoshop (Adobe Systems, San Jose CA). The extent of A-to-I RNA editing was estimated by calculating the proportion of the total height of the both the C and T peaks at each editing site (representing

Table 6.2 Sequences of primers used.

Name	Sequence
DmelActinF	ACTCCGGCGATGGTGTCTCC
DmelActinR	GGGCGGTGATCTCCTTCTGC
Dα6ORF-F	CACGCGATACAAACAAGCCAAGGACA
Dα6ORF-R	ACGATTATGTGCGGAGCGGAGAG
Dα6InternalR2	TGGGCAGCAGGCGTAGACT
Dα6ORF-F3	GCGCCTGCTGAACCATCTGC
Dα6ORF-R3	ACCACCGACGAGGGCGACCAT

edited and unedited transcripts, respectively) that was composed of the height of the C peak as previously described (Jones et al., 2009). The editing rates of flies from each cross were compared separately to the male and female parental strains by a paired t-test in Minitab (Minitab, State College PA).

6.2.4 Insecticides

Spinosad (98% purity) and imidacloprid (99.5%) were obtained from Chem Service (West Chester, PA). Stock solutions of both compounds were dissolved in acetone. All other chemicals were obtained from Fisher (St. Louis, MO).

6.2.5 Bioassays

One to three day old, mixed sex adults were used in both bioassays. The stock solutions of spinosad and imidacloprid were serially diluted in acetone and 10% sugar water (w/v), respectively, to find concentrations of each compound that provided >0% and <100% mortality. A contact bioassay was used to evaluate spinosad toxicity. Scintillation vials with an internal surface area of 38.6 cm^2 (Wheaton Scientific, Millville, NJ) were filled with 0.5 ml of spinosad solution. Plain acetone was used as a control. Vials were placed on their side in a fume hood and rolled to evenly coat the inner surface of the vials. Treated vials were allowed to dry for 1 hr. Flies were placed in a vial covered with white nylon tulle and plugged with a cotton ball. The cotton balls were wetted daily with 10% sugar water.

A feeding bioassay was used to evaluate imidacloprid toxicity. Flies were placed in a scintillation vial that was covered with white nylon tulle and plugged with a cotton ball. Imidacloprid solutions were applied to cotton ball. Plain 10% sugar water was used as a control.

The cotton for all treatments was wetted daily with 10% sugar water. Mortality was assessed after 72 hrs for both spinosad and imidacloprid. Bioassay data were pooled across replicates and analyzed by standard Probit analysis (Finney, 1971), as adapted to personal computer use (Raymond, 1985) using Abbott's correction for control mortality (Abbott, 1925).

The Insensitivity Ratio (IR) and the 95% Confidence Interval (CI) were calculated by standardizing the LC_{50} values of each cross relative to the parental driver and dAdar values (Robertson and Preistler, 1992). The IR values were considered significantly different when the 95% CI did not include 1. RNA-editing in a specific tissue was considered to significantly affect the sensitivity to spinosad and imidacloprid when the IRs were significantly different for both parental comparisons of both reciprocal crosses.

6.3 Results

6.3.1 Estimate of RNA Editing

The proportion of A-to-I RNA editing in all parental strains of *D. melanogaster* is shown in Table 6.3. Editing in all parental strains at each editing site was very similar, but editing was higher in elav3 at all editing sties was higher than all other strains. All crosses resulted in reduced RNA editing of at least one *Dα6* editing site relative to at least one parental strain (Table 6.4). There was variation in the relative suppression of dADAR, as measured by editing of *Dα6*, in each of the crosses. Greatest suppression of editing was seen using the actin driver, while little or no suppression was seen using the cha driver. The dj667 and repo drivers gave intermediate levels of editing suppression in muscle and glia, respectively (Table 6.4). The reduction in editing frequency was not uniform across editing sites (Table 6.4). Editing was completely

Table 6.3 Proportion of RNA editing at 6 editing sites of *Dα6* in parental *D. melanogaster* strains used in this study. Numbers in parentheses indicate standard deviation.

	Editing Site						
Strain	**398**	**400**	**415**	**415**	**468**	**560**	**Average**
actin	0.14 (0.2)	0.13 (0.02)	0.67 (0.04)	0.77 (0.03)	0.81 (0.01)	0.63 (0.03)	0.53 (0.02)
elav	0.10 (0.00)	0.08 (0.02)	0.59 (0.02)	0.72 (0.01)	0.75 (0.03)	0.57 (0.01)	0.47 (0.01)
elav3	0.19 (0.00)	0.20 (0.01)	0.82 (0.02)	0.91 (0.01)	0.94 (0.01)	0.66 (0.00)	0.62 (0.01)
cha	0.10 (0.00)	0.09 (0.01)	0.57 (0.02)	0.72 (0.01)	0.75 (0.01)	0.57 (0.03)	0.47 (0.01)
dj667	0.16 (0.00)	0.14 (0.02)	0.67 (0.01)	0.77 (0.02)	0.81 (0.02)	0.61 (0.01)	0.53 (0.01)
repo	0.10 (0.05)	0.10 (0.01)	0.66 (0.01)	0.81 (0.04)	0.79 (0.02)	0.66 (0.03)	0.52 (0.01)
dAdar	0.13 (0.02)	0.12 (0.01)	0.68 (0.05)	0.79 (0.03)	0.80 (0.01)	0.65 (0.04)	0.53 (0.03)

Table 6.4 Relative amounts of RNA editing in progeny of crosses. Shaded values represent RNA editing rates relative to the parental driver strain and unshaded values represent RNA editing rates relative to dAdar parental. Numbers in parentheses indicate the standard deviation. Values with an * are not different from the parental strain.

	Editing Site						
Cross	**398**	**400**	**415**	**416**	**468**	**560**	**Average**
actin x	0.02 (0.03)	0.00 (0.00)	0.32 (0.13)	0.45 (0.11)	0.58 (0.12)	0.23 (0.13)	0.37 (0.11)
dAdar	0.02 (0.03)	0.00 (0.00)	0.32 (0.13)	0.43 (0.11)	0.59 (0.12)	0.22 (0.12)	0.37 (0.11)
dAdar x	0.00 (0.00)	0.04 (0.05)	0.35 (0.11)	0.44 (0.14)	0.59 (0.09)	0.21 (0.04)	0.38 (0.08)
actin	0.00 (0.00)	0.05 (0.05)	0.35 (0.11)	0.42 (0.14)	0.60 (0.09)	0.20 (0.03)	0.37 (0.08)
elav x	0.08 (0.12)	0.12 (0.08)	0.49 (0.09)	0.64 (0.08)	0.82 (0.09)	0.35 (0.10)	0.56 (0.08)
dAdar	0.06 (0.09)	0.09 (0.01)	0.43 (0.03)	0.58 (0.01)	0.77 (0.02)	0.31 (0.05)	0.50 (0.02)
dAdar x	0.53 (0.09)	0.62* (0.21)	0.74 (0.11)	0.83 (0.07)	0.93* (0.05)	0.68 (0.07)	0.79 (0.08)
elav	0.38 (0.07)	0.43 (0.14)	0.64 (0.09)	0.75 (0.07)	0.87 (0.05)	0.60 (0.06)	0.70 (0.07)
elav3 x	0.21 (0.05)	0.45 (0.16)	0.61 (0.05)	0.73 (0.02)	0.78 (0.08)	0.51 (0.09)	0.63 (0.04)
dAdar	0.30 (0.07)	0.76* (0.27)	0.73 (0.06)	0.84 (0.02)	0.91* (0.09)	0.52 (0.09)	0.74 (0.04)
dAdar x	0.22 (0.09)	0.23 (0.02)	0.49 (0.07)	0.66 (0.03)	0.80 (0.02)	0.36 (0.11)	0.56 (0.05)
elav3	0.32 (0.12)	0.38 (0.04)	0.59 (0.09)	0.75 (0.04)	0.94 (0.02)	0.37 (0.11)	0.65 (0.06)
cha x	1.02* (0.26)	1.10* (0.18)	1.09 (0.04)	1.04* (0.03)	1.05 (0.03)	1.04* (0.07)	1.04* (0.05)
dAdar	0.75* (0.19)	0.78* (0.12)	0.92* (0.04)	0.94* (0.03)	0.97* (0.03)	0.91* (0.06)	0.91 (0.04)
dAdar x	0.94* (0.21)	1.13* (0.13)	1.10 (0.02)	1.04* (0.02)	1.04 (0.01)	1.04* (0.03)	1.05* (0.03)
cha	0.69* (0.15)	0.80 (0.09)	0.93* (0.02)	0.94* (0.02)	0.96 (0.01)	0.91* (0.03)	0.92* (0.02)
dj667 x	0.59 (0.04)	0.61 (0.04)	0.86 (0.05)	0.94 (0.03)	0.97* (0.02)	0.83 (0.07)	0.88 (0.04)
dAdar	0.69 (0.05)	0.71 (0.05)	0.85 (0.04)	0.91 (0.03)	0.98* (0.02)	0.79 (0.06)	0.87 (0.04)
dAdar x	0.53 (0.05)	0.54 (0.08)	0.84 (0.04)	0.93 (0.03)	0.95 (0.01)	0.81 (0.06)	0.86 (0.03)
dj667	0.62 (0.06)	0.62 (0.09)	0.84 (0.04)	0.90 (0.03)	0.95 (0.01)	0.77 (0.05)	0.85 (0.03)
repo x	0.90* (0.06)	0.99* (0.05)	0.94* (0.04)	0.91 (0.01)	0.98* (0.00)	0.91 (0.03)	0.94 (0.02)
dAdar	0.70 (0.04)	0.79 (0.04)	0.92* (0.03)	0.93 (0.01)	0.96 (0.00)	0.93* (0.03)	0.92 (0.02)
dAdar x	0.95* (0.03)	1.00* (0.06)	0.95* (0.03)	0.92 (0.01)	0.99* (0.02)	0.89 (0.02)	0.94 (0.01)
repo	0.74* (0.02)	0.80 (0.05)	0.93* (0.03)	0.94 (0.01)	0.97* (0.02)	0.91* (0.02)	0.93* (0.01)

eliminated at some sites (i.e. site 398 of dAdar x actin), while the editing rate remained at ~60% relative to parental strains (i.e. site 468 of dAdar x actin).

6.3.2 Spinosad Bioassay

The LC_{50} for spinosad in the parental strains ranged from 4.7 ng/cm^2 in elav3 to 35.9 ng/cm^2 in dAdar (Table 6.5). Ubiquitous reduction in RNA editing (via the actin driver) decreased insensitivity to spinosad. Reduction of RNA editing in cholinergic neurons (cha) and muscles (dj667) increased spinosad insensitivity (Figure 6.1). Flies with reduced *dAdar* expression in muscle were more insensitive to spinosad than reduction of *dAdar* expression in cholinergic neurons. There was no significant change in spinosad insensitivity due to reduced editing in the nervous system (elav and elav3) or glia (repo, Figure 6.1).

6.3.3 Imidacloprid Bioassay

The LC_{50} for imidacloprid in the parental strains ranged from 4.2 μg/cotton ball in repo to 30.3 μg/cotton ball in actin (Table 6.5). Reduction in RNA editing in cholinergic neurons (cha), muscles (dj667) and glia (repo) significantly increased imidacloprid insensitivity (Figure 6.2). The most dramatic reduction in imidacloprid sensitivity was seen when *dAdar* expression was reduced in glia. There was no significant change in imidacloprid insensitivity with reduced editing in all tissues (actin) or in the entire nervous system (elav and elav3, Figure 6.2).

Table 6.5 Toxicity of spinosad and imidacloprid to various strains and crosses of *D. melanogaster*.

	Spinosad			Imidacloprid		
Strain	n[a]	LC_{50}[b]	Slope[c]	n[a]	LC_{50}[d]	Slope[c]
actin	1100	25.0 (22.4-28.0)	2.4 (0.2)	505	30.3 (23.5-39.6)	1.8 (0.2)
elav	672	15.1 (12.0-18.5)	1.3 (0.1)	565	6.5 (5.5-7.8)	2.8 (0.4)
elav3	357	4.7 (2.7-6.5)	1.3 (0.2)	289	10.6 (6.4-15.0)	1.6 (0.3)
cha	313	29.3 (24.0-34.2)	2.9 (0.4)	461	18.5 (15.6-22.2)	2.5 (0.3)
dj667	915	11.8 (8.7-28.6)	1.8 (0.5)	512	20.4 (17.6-24.0)	2.6 (0.2)
repo	731	10.8 (9.45-12.2)	2.3 (0.2)	716	4.2 (3.5-5.0)	1.7 (0.1)
dAdar	1349	35.9 (30.6-42.4)	1.3 (0.1)	753	23.2 (20.3-26.7)	2.1 (0.2)
actin♀ x dAdar♂	340	6.1 (4.1-8.12)‡*	1.8 (0.2)	770	15.0 (12.6-17.3)‡*	1.9 (0.2)
dAdar♀ x actin♂	393	14.9 (9.8-20.6)‡*	1.5 (0.2)	647	28.2 (24.5-32.0)	2.3 (0.2)
elav♀ x dAdar♂	355	19.3 (14.6-23.7) *	1.7 (0.2)	717	17.7 (15.3-20.6) ‡	2.1 (0.2)
dAdar♀ x elav♂	378	48.7 (43.1-54.5) ‡*	2.7 (0.2)	965	18.9 (16.3-21.9) ‡	2.1 (0.1)
elav3♀ x dAdar♂	614	12.7 (11.3-14.4)	2.6 (0.2)	881	27.1 (24.2-29.9) ‡	3.9 (0.3)
dAdar♀ x elav3♂	707	13.2 (11.8-14.9)	2.4 (0.2)	1060	23.2 (21.3-25.3) ‡	3.3 (0.2)
cha♀ x dAdar ♂	405	43.4 (35.1-52.8) ‡	1.8 (0.2)	345	42.1 (38.0-46.3) ‡*	4.1 (0.5)
dAdar♀ x cha♂	484	75.5 (61.9-97.5) ‡*	1.6 (0.2)	371	35.5 (30.5-41.1) ‡*	2.4 (0.3)
dj667♀ x dAdar♂	480	70.6 (60.9-82.1) ‡*	2.3 (0.4)	548	38.2 (30.1-52.0) ‡*	1.7 (0.2)
dAdar♀ x dj667♂	389	58.8 (48.7-68.3) ‡*	2.4 (0.4)	446	47.1 (36.7-66.3) ‡*	1.7 (0.3)
repo♀ x dAdar♂	348	42.5 (35.0-48.7) ‡	3.3 (0.5)	223	49.6 (39.2-64.7) ‡*	2.5 (0.4)
dAdar♀ x repo♂	447	31.0 (25.9-36.0) ‡	2.4 (0.2)	251	61.8 (47.8-82.1) ‡*	2.2 (0.3)

a = number of flies per treatment

b = units in ng/cm^2, numbers in parenthesis is the range of the LC_{50}

c = number in parentheses are standard error of the slope

d = units in mg/cotton ball, numbers in parenthesis is the range of the LC_{50}

‡ = LC_{50} significantly different than parental driver

* = LC_{50} significantly different than dAdar

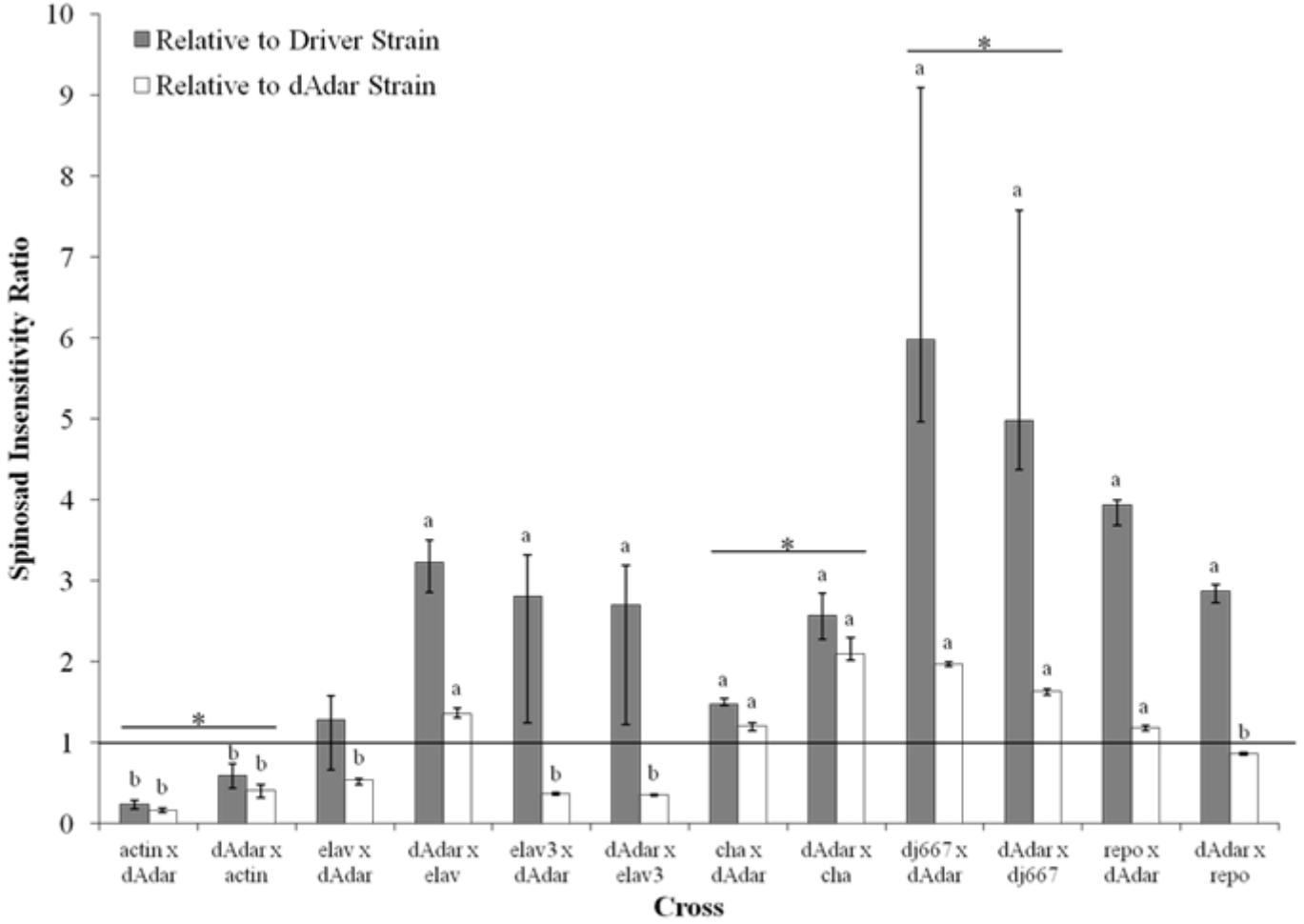

Figure 6.1 Changes in spinosad insensitivity due to reduction of A-to-I RNA editing activity in specific tissues of *D. melanogaster*. Columns represent the insensitivity ratios and bars represent the standard deviation of the insensitivity ratio. Insensitivity ratios significantly greater than or less than 1 are indicated by "a" or "b", respectively. If both reciprocal crosses showed the same result (i.e. significantly greater or less than 1) relative to both parental strains, they were deemed to have a significant driver specific effect indicated by an asterisk.

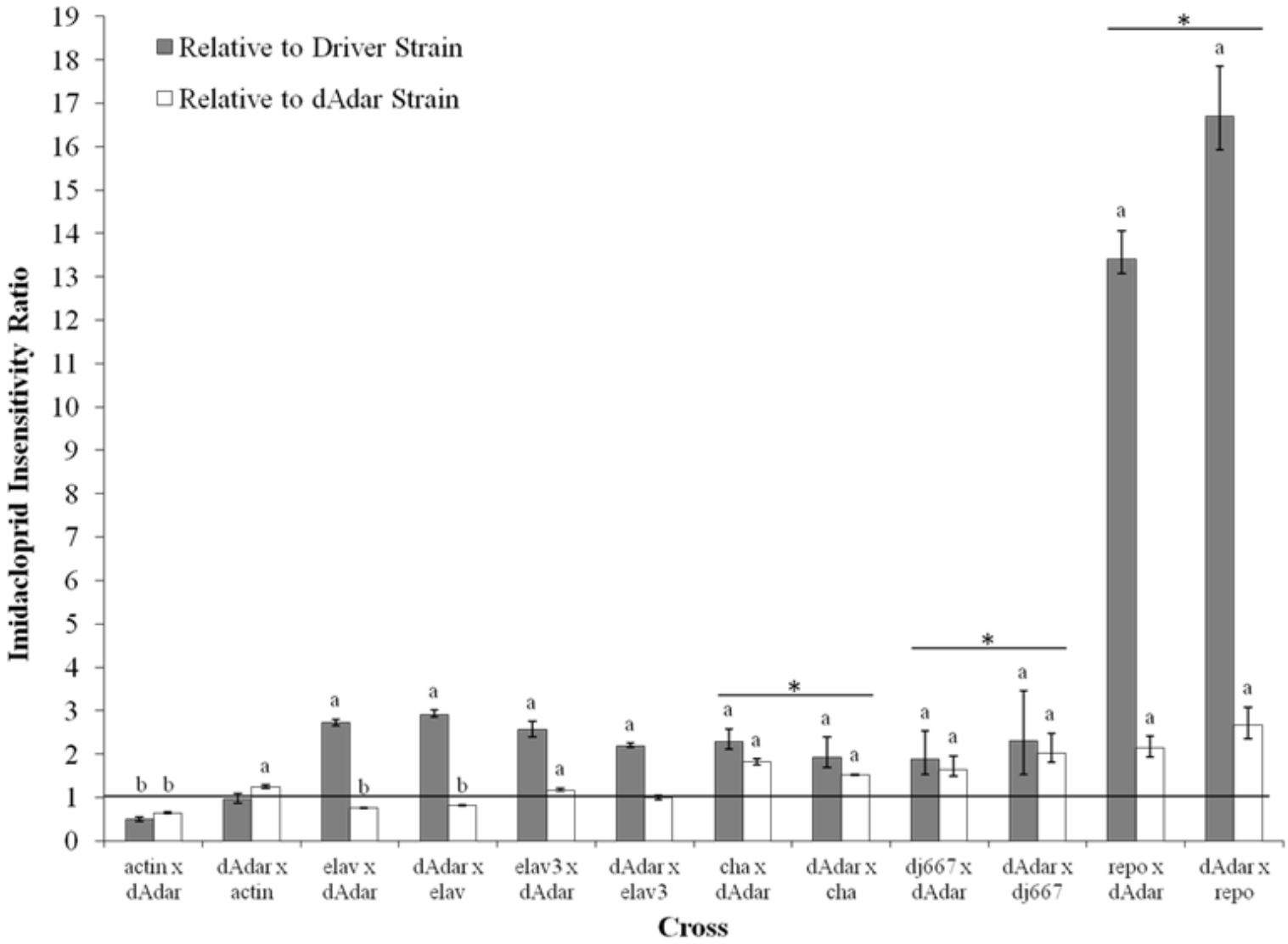

Figure 6.2 Changes in imidacloprid sensitivity due to reduction of A-to-I RNA editing activity in certain tissues of *D. melanogaster*. Significant insensitivity ratios are indicated as described in Figure 6.1.

6.4. Discussion

6.4.1 Tissue-Specific Reductions in A-to-I RNA Editing

The rate of A-to-I RNA editing varied based on the tissue in which editing was reduced and which editing site was evaluated. In fact, editing site 468 across all crosses was the most resistant to reduction in editing rate, which is in agreement with previous work (Figure 2E in (Jepson and Reenan, 2009)). The difference in editing reduction is likely due to the different editing efficiency of these sites and not to the reduction in dADAR expression, as extensive editing may be possible with a very small level of dADAR. The differences in editing efficiency may be due to differences in the structure of dsRNA needed for dADAR activity (Jepson et al., 2011). Because of this difference in editing efficiency, it is unclear which site(s) is most important for changes in insecticide sensitivity *in vivo*. Heterologous expression of these receptors with the appropriate combinations of edited sites will provide more specific information on the role of A-to-I RNA editing on insecticide sensitivity. This future course of study is promising as it has been demonstrated that editing of *Nlβ1* reduces sensitivity of the receptor to imidacloprid, but not to acetylcholine (Yao et al., 2009).

6.4.2 Changes in Spinosad Insensitivity

Ubiquitous reduction in *dAdar* expression decreased the insensitivity to spinosad. Reduction in editing of genes expressed outside of the nervous system causes the decreased insensitivity of spinosad. This is because reduced *dAdar* expression in the entire nervous system (elav and elav3 drivers) did not significantly alter spinosad insensitivity, while ubiquitous reduction of *dAdar* expression (actin driver) decreased spinosad insensitivity (Figure 6.1). The specific genes that may be involved in the decrease in spinosad insensitivity are unknown as

spinosad primarily targets the nAChR (Salgado, 1998, Salgado and Sparks, 2005, Salgado et al., 1998) and has a secondary target site at GABA receptors (Watson, 2001).

It has been convincingly demonstrated that *Dα6* and *Pxylα6* define the target site of spinosad (Perry et al., 2007, Watson et al., 2010, Rinkevich et al., 2010). Therefore, it is likely that reduced editing of *Dα6* in cholinergic neurons results in the decrease of spinosad sensitivity. The reduction in editing in cholinergic neurons does not dramatically reduce the level of RNA editing (Table 6.3), therefore, it is a very specific editing event (i.e. editing of *Dα6*) in cholinergic neurons that causes the reduction of spinosad sensitivity. *Dα6* is edited at 7 sites in the ligand binding loops E, B and F (Grauso et al., 2002). However, it has been shown that spinosad does not interact with the receptor in the ligand binding domain in the same manner as traditional agonists (Orr et al., 2009, Salgado and Saar, 2004).

Surprisingly, reduced editing in muscle (via dj667 driver) caused the most significant shift in spinosad insensitivity (Figure 6.1). Total editing of *Dα6* was reduced to 85-88% relative to the parental strains. This reduction in editing suggests that *Dα6* is also expressed in muscle. This is an unexpected result because it is thought that nAChRs, such as *Dα6* which is the target of spinosad, have previously been thought to be only expressed in the nervous system (Sattelle et al., 2005). This also suggests that spinosad may have an additional non-nAChR target site in the muscle. GABA receptors have been identified as secondary targets of spinosad, but those receptors were identified from small diameter neurons (<20 μm) of abdominal ganglia where spinosad was antagonistic (Watson, 2001). Additionally, GABA receptors composed of *Rdl* subunits are restricted to the CNS (Buckingham et al., 2005), although GABA-induced currents are found in muscle (Rauh et al., 1997, Schnee et al., 1997). These other GABA receptor subunits, GRD and LCCH3, may be the secondary targets of spinosad in the muscle. Receptors

containing *Rdl* and LCCH3 subunits possess pharmacology and physiology that is distinct from *Rdl* homopentameric receptors (Zhang et al., 1995). However, the GABA receptor subunit composition with which spinosad interacts is unknown.

6.4.3 Changes in Imidacloprid Insensitivity

Imidacloprid sensitivity was significantly reduced when expression of *dAdar* was reduced in cholinergic neurons, muscle, and glia. Imidacloprid insensitivity has been demonstrated with a Y151S mutation in *Nlα1* of the brown planthopper (Liu et al., 2006). Heterologous expression of receptors with an N133D editing substitution in *Nlβ1* results in reduced imidacloprid potency (Yao et al., 2009). Lesions in *Dα1* and *Dβ2* cause reduced sensitivity to several neonicotinoids in larval feeding bioassays (Perry et al., 2008). While *Dβ1* and *Dβ2* are edited, only *Dβ1* is edited in the ligand binding domain in loop D (Sattelle et al., 2005). Amino acids in and around loop D are important for ligand interactions (Liu et al., 2008, Shimomura et al., 2003, Toshima et al., 2009). Therefore, editing of *Dβ1* is likely to be responsible for the reduction in imidacloprid sensitivity. This scenario is plausible for expression in cholinergic neurons because of the expression pattern of nAChRs (Sattelle et al., 2005). It does not however, lend itself easily to explain the reduced imidacloprid sensitivity when *dAdar* is reduced in muscle, as glutamate, rather than ACh, is the excitatory neurotransmitter at the insect neuromuscular junction.

The observation of increased imidacloprid insensitivity in glia is an unexpected result. Glia serve a myriad of functions for neurons such as supplying nutrients, guiding axonal development, and synaptic modulation (Edwards and Meinertzhagen, 2010). Olfactory receptor axons release ACh that cause ACh-inducible Ca^{2+} currents which are blocked by nAChR antagonists in glia in pupae of the tobacco hornworm, *Manduca sexta*. These currents are

necessary and sufficient to cause migration of glial cells. The presence of ACh-inducible Ca^{2+} currents indicates nAChRs are expressed in glia (Heil et al., 2007). The fact that such a large change in imidacloprid sensitivity is seen when *dAdar* expression is reduced in glia, indicates glial functions that are modified by A-to-I RNA editing are important features of the interaction of imidacloprid with the nAChR. While the direct interaction of imidacloprid with *Dα1*, *Dβ1* and *Dβ2* containing receptors has been clearly demonstrated (Perry et al., 2008, Yao et al., 2009), these results indicate the extent by which other interactions may play a role in the larger picture of imidacloprid toxicity *in vivo*. These other interactions may not be as important to overall toxicity as direct interaction with the receptor because the changes in toxicity were ~2 to 17 fold relative to the parental strains (Figure 6.2), whereas mutations in receptor subunits cause up to 250-fold resistance (Liu et al., 2005).

6.4.4 Tissue-Specific Changes of Insecticide Insensitivity

The results indicate that A-to-I RNA editing is an important process that influences the sensitivity to spinosad and imidacloprid *in vivo*. Spinosad insensitivity is decreased by reduced ubiquitous editing, but increased by reduced editing in cholinergic neurons and muscle, indicating that tissue specific editing can produce variable responses to spinosad insensitivity. There was no significant change in spinosad insensitivity by reducing editing in glia, but the greatest change in imidacloprid insensitivity was observed with reduced editing in glia. This indicates spinosad and imidacloprid may target different tissues.

Reduction in *Dα6* editing in all tissues and the nervous system was consistent with the pattern of both *dAdar* and *Dα6* expression (Palladino et al., 2000a, Sattelle et al., 2005). There is very little *Dα6* editing in cholinergic neurons (Table 6.3), although flies with reduced editing in

cholinergic neurons were less sensitive to both spinosad and imidacloprid (Figure 6.1 and Figure 6.2). Editing of *Dα6* is reduced in both the muscle and glia, which stands in stark contrast to the idea that nAChRs are only expressed in the excitatory central nervous system (Sattelle et al., 2005). Although reduction of *dAdar* expression in glia driven by the Gal4-UAS system can result in changes in phenotype (Jepson and Reenan, 2009, Keegan et al., 2005), dADAR did not co-localize with the glial nuclear marker repo (Jepson et al., 2011). Therefore, it is difficult to connect the change in editing frequency with the reduction of *dAdar* expression driven by repo when immunohistochemistry shows that dADAR is not expressed in glia.

We have demonstrated that A-to-I RNA editing influences the sensitivity to two classes of insecticides that interact at the nAChR. Some of these changes in toxicity were somewhat unexpected as the results indicate a broader range of potential target sites for these insecticides, and other processes may have a greater influence on insecticide sensitivity than previously thought. It will be necessary to determine which specific transcripts that undergo A-to-I RNA editing are important for influencing insecticide sensitivity. These results provide a strong foundation to assess the role of A-to-I RNA editing of nAChR subunits on insecticide sensitivity, especially in the case of spinosad and *Dα6*.

6.5 Acknowledgements

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Chapter 7

A Rapid, Sensitive, and Cost-Effective Method for Estimating the Frequency of A-to-I RNA Editing[3]

7.1 Introduction

A-to-I RNA editing is catalyzed by adenosine deaminases that act on RNA (ADARs) that bind to double stranded pre-mRNAs and convert adenosine (A) to inosine (I) which is recognized by the ribosome as guanosine (Nishikura, 2010). ADARs are found in all animals, but are absent from protists, plants and fungi (Jin et al., 2009). There are three ADAR genes in vertebrates. *ADAR1* and *ADAR2* both have catalytic activity, whereas *ADAR3* lacks activity, although the functional domains of *ADAR3* are conserved. *ADAR3* is likely is a duplicate of *ADAR2*. A single ADAR gene exists in insects and *Drosophila melanogaster dAdar* is homologous to vertebrate *ADAR2* (Jin et al., 2009).

A-to-I RNA editing regulates behavior and life history traits in many phyla of animals. The widespread conservation of this pathway is thought to be a viral defense mechanism (Bass and Weintraub, 1988, Bass, 2002). A-to-I RNA editing occurs in protein coding and non-coding sequences, transposable elements, introns, 5′ and 3′ untranslated regions of the pre-mRNA that may result in changes in the amino acid sequences, splice sites or levels of transcripts (Bass, 2002, Fukui and Itoh, 2010). RNA editing frequently results in non-synonymous substitutions that can be critically important for proper function or tissue distribution. For example, RNA editing results in a Q/R amino acid substitution in the pore loop domain of human GluR-B that makes the channel impermeable to Ca^{2+}. Unedited GluR-B transcripts lead to neuronal death that causes seizures and premature death in editing deficient mice (Higuchi et al., 2000).

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The transcripts of many ligand-gated or voltage-sensitive ion channels and G-protein coupled receptors are targets of A-to-I RNA editing (Bass, 2002, O'Connell, 1997, Paul and Bass, 1998, Hoopengardner et al., 2003). Genome wide studies in *D. melanogaster* have shown wide-spread editing of these genes (Hoopengardner et al., 2003). RNA editing of these genes is most common in regions that code for functionally important amino acids in the protein. In voltage-gated K^+, Na^+ and Ca^{2+} channels, residues involved in channel gating or inactivation are edited. Editing sites on nAChR or GABA receptor subunits occur in crucial areas in the ligand-binding domain and TM2 that forms the channel pore (Hoopengardner et al., 2003, Jones et al., 2009, Yao et al., 2009, Song et al., 2004).

There are a number of *dAdar* transcripts produced and *dAdar* expression is important for a number of functions in the fruit fly. Four common transcripts of *dAdar* result from alternative splicing and these transcripts are themselves subject to RNA editing (Palladino et al., 2000a). *D. melanogaster dAdar* null mutants show significant deficiencies in motor control and mating that grow progressively worse with age. Nervous system morphology is greatly affected by *dAdar* null mutants (Palladino et al., 2000b). Behavioral deficits were also seen in *D. melanogaster* adults that have reduced *dAdar* expression. These flies did not fly or exhibit diurnal activity patterns, and displayed temperature sensitive paralysis (Jepson and Reenan, 2009).

There are a variety of methods for measuring the extent of A-to-I RNA editing (Jepson and Reenan, 2007, Abbas et al., 2010) and these are important for the burgeoning study of A-to-I RNA editing (since 2007, there have been more than 1080 journal articles, book chapters and meeting presentations on RNA editing). However, current methods to measure the extent of RNA editing tend to be costly, time consuming and use radioactive materials or generate hazardous waste. Using the *Dα6* nicotinic acetylcholine receptor subunit, I demonstrate a

detailed and improved method for a highly accurate and precise estimate of editing rates using peak height ratios from Sanger sequencing electropherograms and compare its costs and benefits to other methods for measuring RNA editing rates.

7.2 Materials and Methods

7.2.1 RNA isolation, *Dα6* PCR, Cloning and Sequencing

Total RNA was isolated from 3 batches of 10, 1-3 day old Canton-S flies (5♀ and 5♂) using TRIzol (Invitrogen, Carlsbad CA) according to the manufacturer's directions. The RNA was dissolved in 50 ul of DEPC-treated water. Reverse transcription was performed with 5 ug of total RNA using Go-Script (Promega, Madison WI) according to the manufacturer's directions.

A 1502 bp fragment comprising the open reading frame of the *Dα6* nicotinic acetylcholine receptor subunit was amplified in 100 ul Go-Taq reactions with 2 ul of cDNA and 2 ul of each primer Dα6ORF-F and Dα6ORF-R (Table 7.1). A control reaction with a 497 bp fragment of *actin5c* was performed under the same reaction conditions with the primers DmelActinF and DmelActinR (Table 7.1). Thermocycler conditions were as follows: 95^{o}C for 2 min, 40 cycles of 95^{o}C for 30 s, 56^{o}C for 30 s, and 72^{o}C for 1.5 min, with a final extension at 72^{o}C for 10 min. PCR products were visualized on a 1% agarose gel stained with 50 ug/ml of ethidium bromide under UV light. PCR products were purified using the Wizard PCR Purification Kit (Promega, Madison WI). Up to 100 ng of the purified *Dα6* open reading frame cDNA were cloned into pGEM-T vector (Promega, Madison WI) according to the manufacturer's directions, except that ligation was carried out at 4^{o}C overnight. JM109 competent cells were transformed via heat shock with 2 ul of the cloning reaction and spread on Luria Beranti plates containing ampicillin (100 ug/ml), X-Gal (40 ug/ml) and ITPG (100 uM)

Table 7.1 Sequences of primers used.

Primer Name	Sequence
Dα6ORF-F	CACGCGATACAAACAAGCCAAGGACA
Dα6ORF-R	ACGATTATGTGCGGAGCGGAGAG
DmelActinF	ACTCCGGCGATGGTGTCTCC
DmelActinR	GGGCGGTGATCTCCTTCTGC
T7	TAATACGACTCACTATAGGG
SP6	TATTTAGGTGACACTATAG
Dα6R	CCAGGGCAGCCATTGTAGGAAAAC
Dα6IR2	GCAGCAGGCGTAGACTATCGTATT
Dα6285F	AACGGAATACGGCGGGGTCAAG
Dα6ORF-F3	GCGCCTGCTGAACCATCTGC
Dα6ORF-R3	ACCACCGACGAGGGCGACCAT

and grown at 37°C overnight. White colonies were screened for the presence of the correct insert by spotting the colony to a fresh plate with a 10 ul pipette tip and using the pipette tip directly in a 15 ul Go-Taq reaction with T7 and SP6 primers (Table 7.1). The conditions for colony PCR were as follows: 95°C for 2 min, 35 cycles of 95°C for 30 s, 42°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. PCR products were visualized on a 1% agarose gel stained with 50 ug/ml of ethidium bromide under UV light. Positive colonies were incubated at 37°C overnight in 3 ml of Luria broth medium containing 100 ug/ml ampicillin. Plasmid DNA was isolated using the Wizard Plus SV Minipreps (Promega, Madison WI) and eluted in 100 µl H_2O. Positive clones were sequenced from both ends using T7, SP6, and Dα6R primers (Table 7.1) at Cornell's Biotechnology Resource Center on an Applied Biosystems Automated 3730 DNA Analyzer using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. Sequences for each clone were aligned using MegAlign (DNA Star, Madison, WI).

7.2.2 Validation of Peak Height Ratio Method Using Clones

Dα6 clones that were entirely edited or unedited at editing sites 398, 400, 415, and 416 (based on the nucleotide numbering of the open reading frame of *Dα6*) were identified (Grauso et al., 2002). I did not observe editing at site 413 in any samples (n=17) in these experiments. Different amounts (by weight) of plasmid DNA from clones that were completely edited or unedited were added to sequencing tubes to provide known ratios of edited and unedited sites to assess 5, 10, 25, 50, 75 90, and 95% editing rates. Plasmids were sequenced with the primers Dα6IR2 (antisense) and Dα6285F (sense(Table 7.1)). The heights of the peaks at each editing site were measured using Photoshop Creative Suite 4 (Adobe Systems Inc, San Jose CA) and the ratio of the peak heights was compared to the expected heights (based on the ratio of the clones

used). The accuracy of the estimates was statistically compared with the expected value with a 1-sample t-test vs the expected value. The slope of the expected vs estimated editing rate was calculated using linear regression in Excel.

7.2.3 Validation of Peak Height Ratio Method Using a Known Sample

A fragment of *Dα6* was amplified from three separate cDNA samples from Canton-S flies with the primers Dα6ORF-F3 and Dα6ORF-R3 in 100 ul Go-Taq reactions under the following thermocycler conditions: 95^{o}C for 2 min, 35 cycles of 95^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 30 s, with a final extension at 72^{o}C for 10 min. PCR products were purified and sent for sequencing with the Dα6IR2 primer as described above. Editing estimates were calculated based on peak height ratios as described above. The estimate from this method was compared to the rate quantified from individual clones as described above. The standard deviation from clone counting was determined based on sample size (n=17).

7.3 Results

7.3.1 Determination of Editing with Dα6IR2

Determination of *Dα6* editing at four sites was very accurate and precise when sequenced with Dα6IR2 (Figure 7.1A), and observed values never varied from the expected values by more than 3% (Table 7.2). The slope of the line from a plot of observed vs. expected editing (for each site) was not significantly different from 1 and the r^2 values were 1.0 (Table 7.3). Therefore, the observed frequency of editing is in agreement with the expected frequency of editing at all sites.

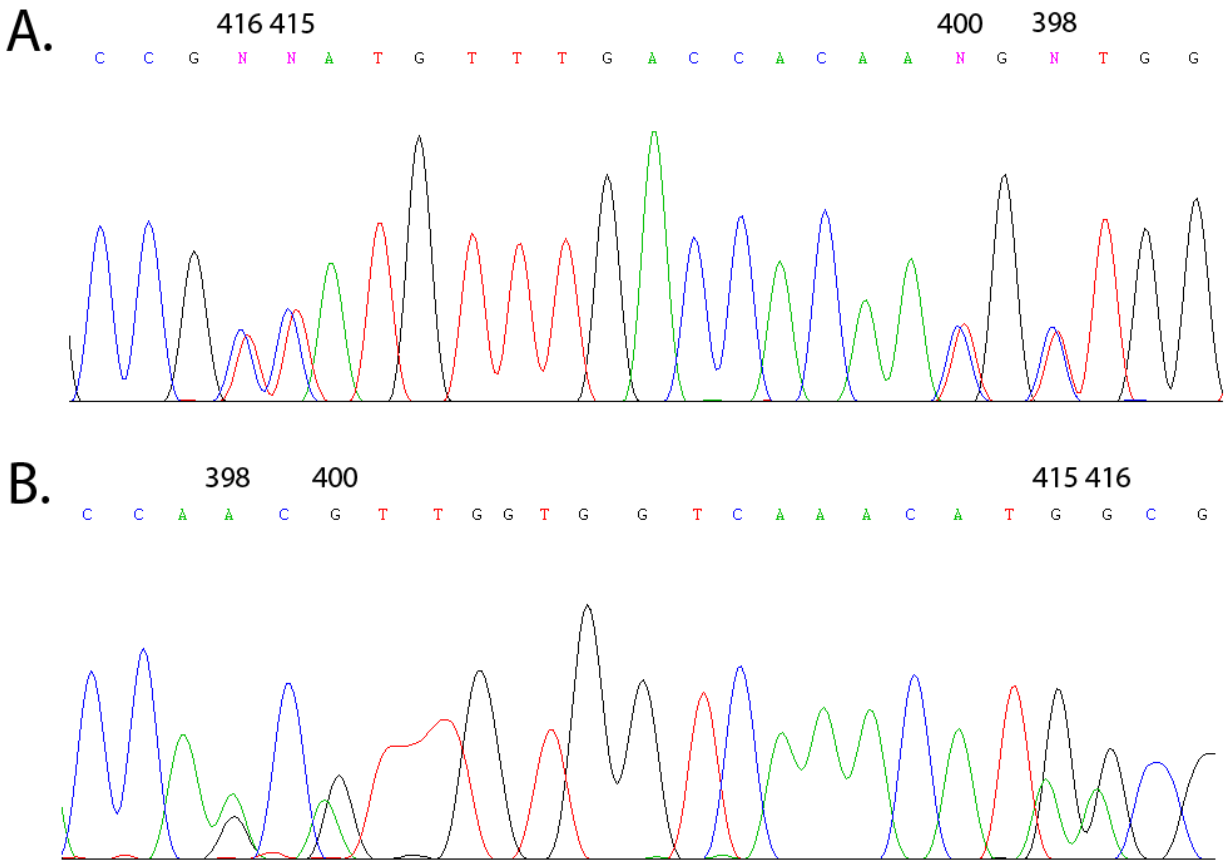

Figure 7.1 Electropherograms representing 1:1 ratios of edited and unedited transcripts sequenced with A) Dα6IR2 (antisense) or B) Dα6285F (sense). Numbers above peaks indicate editing sites based on the nucleotide numbering of the open reading frame of Dα6. Note that Dα6IR2 is an antisense primer so the sequence of A-to-I editing sites is in reverse. Antisense sequencing with Dα6IR2 generates editing sites as a mix of C/T signals, whereas sense sequencing with Dα6285F generates editing sites as a mix of A/G signals

Table 7.2 Estimates of A-to-I RNA editing frequency between Dα6IR2 and Dα6285F sequencing primers. Values represent the mean ± standard deviation. Values with * indicate estimated editing frequency is different from the expected editing rate (one-sample t-test vs expected mean, $p<0.05$).

Sequencing Primer	Expected Editing Rate	Editing Site			
		398	**400**	**415**	**416**
Dα6IR2	0.95	0.94 ± 0.02	0.96 ± 0.01	0.92 ± 0.03	0.92 ± 0.03
	0.90	0.88 ± 0.01*	0.90 ± 0.01	0.87 ± 0.00*	0.88 ± 0.01
	0.75	0.76 ± 0.01	0.74 ± 0.01	0.75 ± 0.02	0.76 ± 0.01
	0.50	0.51 ± 0.01*	0.50 ± 0.03	0.50 ± 0.02	0.52 ± 0.01*
	0.25	0.25 ± 0.03	0.24 ± 0.02	0.23 ± 0.01	0.25 ± 0.02
	0.10	0.11 ± 0.03	0.09 ± 0.01	0.09 ± 0.02	0.09 ± 0.03
	0.05	0.07 ± 0.03	0.07 ± 0.02	0.08 ± 0.05	0.07 ± 0.05
Dα6285F	0.95	0.85 ± 0.12	0.88 ± 0.17	0.97 ± 0.03	0.97 ± 0.02
	0.90	0.86 ± 0.05	0.86 ± 0.05	0.95 ± 0.01*	0.93 ± 0.00*
	0.75	0.66 ± 0.04*	0.79 ± 0.04	0.84 ± 0.03*	0.81 ± 0.03*
	0.50	0.38 ± 0.02*	0.58 ± 0.02*	0.67 ± 0.01*	0.60 ± 0.05*
	0.25	0.19 ± 0.03*	0.33 ± 0.04*	0.43 ± 0.04*	0.39 ± 0.13
	0.10	0.06 ± 0.03	0.17 ± 0.02*	0.33 ± 0.06*	0.23 ± 0.10*
	0.05	0.04 ± 0.02	0.13 ± 0.04	0.25 ± 0.04*	0.23 ± 0.06*

Table 7.3 Comparison of the reliability of estimating A-to-I RNA editing of the Dα6 subunit using the peak height ratio method between Dα6IR2 and Dα6285F primers at different rates of expected editing. Values are the slopes and associated confidence intervals of editing estimate vs. expected values. Numbers in parentheses indicate the r^2 value. Slopes with * indicate values that are significantly different than 1 (i.e., 95% CI does not include 1).

	Editing Site			
Seq. Primer	**398**	**400**	**415**	**416**
Dα6IR2	0.98 ± 0.03 (1.00)	1.00 ± 0.03 (1.00)	0.97 ± 0.05 (1.00)	0.97 ± 0.05 (1.00)
Dα6285F	0.94 ± 0.10 (0.99)	0.88 ± 0.10* (0.99)	0.80 ± 0.07* (0.99)	0.85 ± 0.04* (1.00)

7.3.2 Determination of Editing with Dα6285F

Determination of *Dα6* editing estimates was unreliable (inaccurate and imprecise) when sequenced with Dα6285F at all four editing sites (Figure 7.1B, Table 7.2). The observed frequency of editing was significantly different than expected in 17 of 28 comparisons and varied by as much as 23% (Table 7.2). In all, 20 estimates were significantly different from the expected editing rate by more than 5%. The slope of the line from a plot of observed values of editing vs. those expected was significantly different from 1.0 for three of the four editing sites and the r^2 value was less than 1.0 in 3 of the 4 cases (Table 7.3). This indicates use of the Dα6285F primer is not a reliable method for determining the frequency of editing.

7.3.3 Validation of the Peak Height Ratio Method Using a Known Sample

The frequency of editing at 4 sites was determined for a sample of Canton-S cDNA by examining the sequences of individual clones determined with the Dα6IR2 primer. This sample was then evaluated using the peak height ratio method. Comparison of these methods showed they were in close agreement and were not significantly different at any editing site (Figure 7.2). This agreement was observed for sites that had either a low (398 and 400) or high frequency of editing (415 and 416).

7.4 Discussion

I have developed and validated a very accurate, precise, fast and cost-effective method for estimating editing rates. The accuracy and precision of the editing estimates were primer specific (Figures 7.1, 7.2; Tables 7.2, 7.3). Results with primer Dα6IR2 were excellent, while those with primer Dα6285F were much less reliable (Figure 7.1). When sequenced with

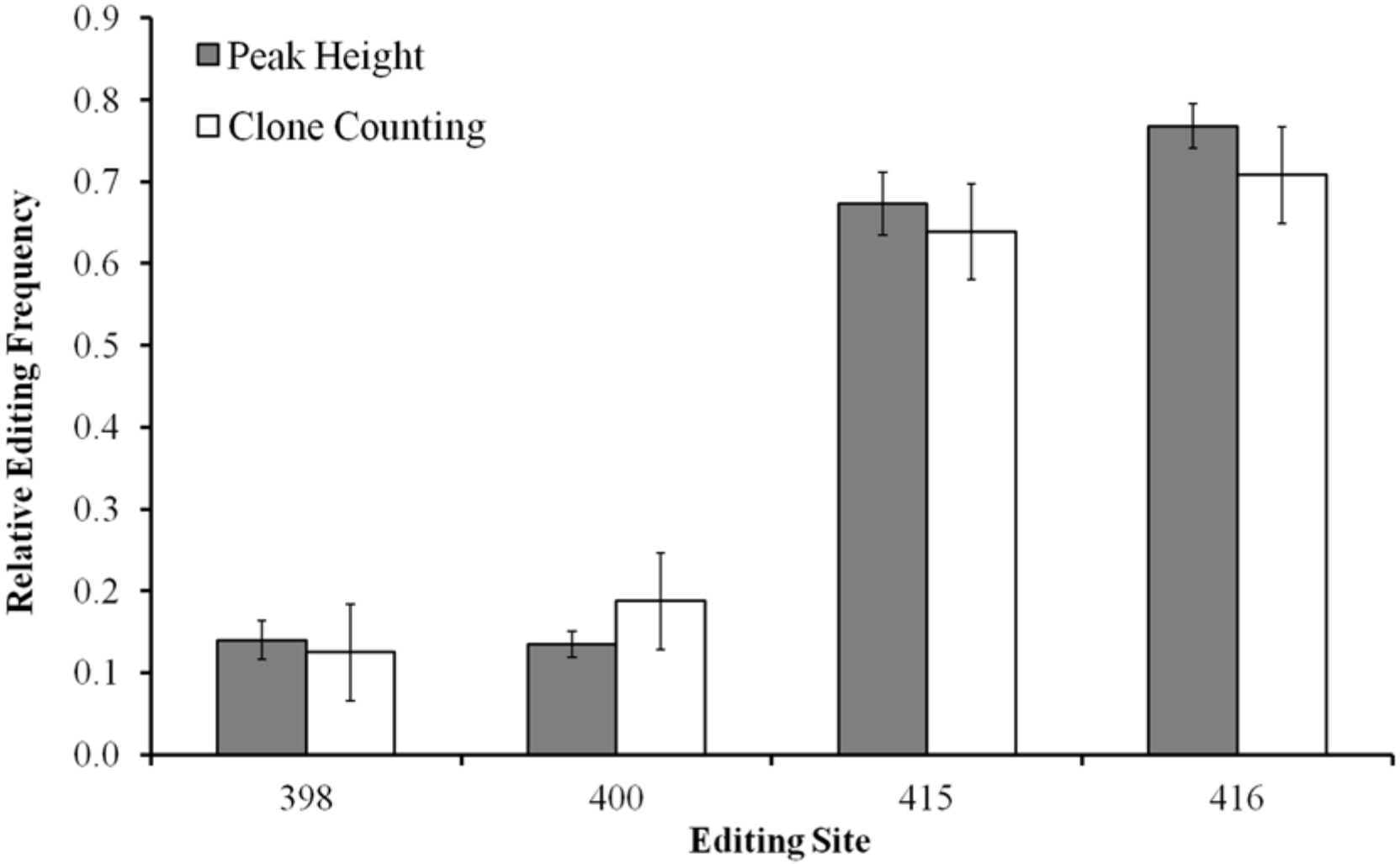

Figure 7.2 Editing estimate comparison between peak height ratios and clone counting at four editing sites of *Dα6*. There is no difference in the editing proportion of samples using the peak height ratio method or clone counting. The editing site numbers represent the base of the open reading frame of *Dα6* that is edited.

Dα6IR2, unedited and edited sites appeared as a mix of T/C signals, respectively. In contrast, results with Dα6285F showed unedited and edited sites as A or G, respectively (Figure 7.1B, Table 7.2). Therefore, it is imperative that the proper primer (Dα6IR2 in this case) be used to accurately estimate the rate of RNA editing.

The disparity in editing estimation is due to the sequencing protocol. The dye-terminator sequencing kits used at Cornell's Biotechnology Resource Center contains dITP instead of dGTP to reduce compression artifacts in the electropherograms. The disadvantage of using dITP in the sequencing reactions is that it is not incorporated as efficiently as dGTP, thus, looking at the A/G (or I due to editing) ratio may be skewed (Innis et al., 1988).

Table 7.4 shows a comparison of the time, labor and financial costs of the peak height ratio method with existing protocols. The financial cost savings are the most significant and noteworthy advantage of the peak height ratio method. The only method that is as inexpensive for a single editing site in a single sample is the fluorescent restriction enzyme digestion. The cost of the clone counting method is incurred by the large number of reps needed to generate a single data point for a biological sample. The high cost of the restriction digest with radioisotopes and poisoned primer extension is mainly due to the high cost of fluorescent dyes and ^{32}P. These two protocols are economically considerable only when larger numbers of reps can be performed in a short period of time. Ultra high throughput sequencing is extremely cost prohibitive and better suited for experiments that may not require comparing many biological samples.

Another major benefit (which can result in additional savings) of this new method is that nearby editing sites can be evaluated simultaneously (Table 7.4). For example, the cost to

Table 7.4 Comparison of methods used to measure A-to-I RNA editing. Costs are based on using the materials listed in the Materials and Methods. Additional costs were based on pGEM-T Cloning System II, PureYield Plasmid Purification System (Promega), restriction enzymes (PshAI and HpyCH4V, New England Biolabs), KinaseMax Kit, Alexa Fluor 488 and NucAway (Invitrogen). Data for Ultra High Throughput Sequencing is based on Abbas et al., 2010 and consists of a single lane 86 bp single-end read on a Genome Analyzer. The cost/sample is the cost of performing a single experiment from one biological sample. The cost/data point is the cost for three replicates from one biological sample. Relative cost is for assessing the four editing sites of *Da6* reported herein. The price of shipping, primers, gels, standard markers, imaging equipment, software, and labor were not included in the cost. Relative cost is for assessing the four editing sites of *Da6* reported herein.

Method	# Steps	Days	Cost/Sample	Cost/ 3 Analyses[a]	Relative Cost	Sites/ Analysis
Peak Height Ratio	6 (RNA isolation, RT, PCR, PCR purification, sequencing, analysis)	3	$11.87	$35.62	1	Several
Poisoned Primer Extension	7 (RNA isolation, RT, primer labeling, PCR, gel, imaging, analysis)	1-2	$120.63 F[b] $123.40 ^{32}P	$133.24 F[b] $157.84 ^{32}P	15.0 F[b] 17.7 ^{32}P	One
Restriction Digest	9 (RNA isolation, RT, PCR, purification, RE digestion, purification, gel, imaging, analysis)	2	$10.37 F[b] $123.40 ^{32}P	$31.10 F[b] $135.70 ^{32}P	3.50 F[b] 15.2 ^{32}P	One
Ultra High Throughput Sequencing	9 (RNA isolation, RT, PCR(x2), purification(x2), hybridization, sequencing, analysis)	21-28	$1609.80	$4829.39	136	One to Several[c]
Clone Counting	10 (RNA isolation, RT, PCR, PCR purification, cloning, transformation, colony screening, colony growth, plasmid purification, sequencing, analysis)	6	$24.48	$561.25	15.8	Several

[a] = Cost/3 Analyses is simply not three-fold the Cost/Rep as primers can be labeled and used in multiple samples on a single day of experiments.
[b] = Fluorescent assay
[c] = the ability to read several editing sites is determined by proximity of editing sites

measure the 4 editing sites of *Dα6* by poisoned primer extension would be 15 to 17 fold higher than by my method (Table 7.4).

My method requires only 6 steps required to generate data from a sample. Every other method requires more steps, all of which can introduce data variation and experimental failure. The turn-around time of the new method is comparable to the restriction digest and poisoned primer extension, shorter than clone counting, and much faster than ultra high throughput sequencing. Another major advantage of the peak height ratio method is that little laboratory infrastructure is required other than what is normally available in most labs. Aside from the kits, enzymes and disposable labware required, the only pieces of equipment needed are a centrifuge and a thermocycler. These data are produced and analyzed *in silico*, which makes data management easier. This amount of technical efficiency makes it an ideal method for demonstrating the extent of A-to-I RNA editing in high school and introductory biology classes, or in laboratories as a significant cost reduction method. Additionally, a mobile lab unit could be assembled for rapid sample processing in the field and other remote areas. The RNA extraction, reverse transcription and PCR could be done in the field and processed samples could be mailed to a sequencing facility. Processing field collected animals would help overcome potential changes in allele frequency that may result from genetic drift if they were returned to a lab and reared for a second generation. This would also reduce the risk of sample degradation and shipping nucleic acids across international borders is typically less burdensome than shipping organisms or tissues.

This method could also be used to estimate allele frequencies within populations. Using pools of animals, it is possible to simultaneously evaluate allele frequencies from as many as 10 diploid or 20 haploid individuals based on the upper (0.95) and lower (0.05) detection rates that I

used in this analysis. This would allow for the detection of a single allele out of 20 potential alleles. It is likely that this method could be validated for even lower detection rates. This method would be extremely valuable in my lab for evaluating the frequency of insecticide resistance alleles from field collected populations.

Besides these savings of time and money, there are many other technical advantages of using peak height ratios over other methods. RNA editing may introduce or eliminate a restriction enzyme site. Poisoned primer extension utilizes ddGTP as a reaction terminator in edited transcripts. A larger band will result from unedited transcripts because dATP would be incorporated into the product. These methods utilize fluorescent or phosphoimaging systems for quantification.

The major drawbacks to poisoned primer extension and restriction digests are that they only allow for the quantification of a single editing site, they use radiolabeled or fluorescently labeled primers, and are somewhat labor intensive and slow. In the case of restriction enzyme digestion, the edited site may alter a restriction enzyme recognition sequence for which an inexpensive or widely used restriction enzyme may not be readily available. In the case of adjacent or nearby editing sites, use of multiple enzymes would be required to account for variation in recognition site. Complications may arise in cases when a restriction enzyme is not available to recognize the change in recognition sequence.

A major limitation of the poisoned primer extension method is that is can only assess one editing site at a time. In order to obtain the same amount of data across four editing sites as used in this experiment, four unique reactions would need to be run on each sample. This would require extensive sample planning and management. Also, using sense primers to asses editing at adjacent editing sites would be particularly troublesome, as in the case of editing sites 415 and

416 of the *Dα6* nAChR subunit used in this experiment. Two sense primers would have to be utilized to account for transcripts with edited and unedited versions at site 415 in order to accurately assess editing at site 416 as mismatches at the 3′ end of primer with template can lead to reduced amplification efficiency. Conversely, an antisense primer could be used to assess editing at site 416 without regard for editing status at site 415. However, this would require the use of ddCTP as the reaction terminator.

Clone counting can be performed to quantify editing rates. While there are many advantages to this method, the major drawback is that a large number of clones need to be sequenced to ensure an accurate reflection of editing rates. This process may take a few days to complete and the cost of sequencing a large number of clones may be substantial. Screening colonies for positive inserts usually requires screening many more colonies than will actually go for sequencing. The waste generated by growing colonies on plates and in liquid media needs to be properly disposed by autoclaving or incinerating at an approved facility.

Next generation high throughput sequencing is the most accurate method to measure RNA editing rates. It can even detect rare transcripts that are missed by clone counting methods (Abbas et al., 2010). The major disadvantages are cost and the short reads generated may only be useful for multiple editing sites that are nearby if the user needs to know what sites are edited on a specific transcript. However, the undeniable major advantage of UHTS is that many editing sites on many transcripts of many genes can be evaluated.

The peak height ratio method utilizes the different intensities in the signal of T/C on chromatograms to asses editing rate. The disadvantage of this method is that it does not allow for the identification of which editing sites are edited on each transcript. However, the advantages in the cost, labor, and turnaround time are unequivocal. The peak height ratio method is very

advantageous in that there are few steps required to complete an analysis from biological sample to data point.

The accuracy and precision of the estimate of A-to-I RNA editing using the peak height ratio method with sequences from the Dα6IR2 primer is in very good agreement with expected values and is comparable to the quantitative clone counting method. It is also very cost effective and fast compared to other current methods, especially when evaluating editing at multiple sites. Because of these many advantages, it is likely that this method will prove to be a powerful and useful tool in evaluating the extent of A-to-I RNA editing in this growing field of study.

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Chapter 8

Future Directions

8.1 Spinosad Resistance Mechanisms in Field-Selected Resistant Insects

Laboratory selection for spinosad resistance has been demonstrated in many insects (Shono and Scott, 2003, Zhao et al., 2002, Bielza et al., 2007, Wyss et al., 2003, Sayyed et al., 2004). The pattern of inheritance for spinosad resistance in these insects is very similar (i.e., recessive, autosomal, monofactorial and unable to be overcome with metabolic inhibitors), thus suggesting a common spinosad resistance mechanism. However, in the case of house fly, spinosad resistance is not due to changes in *Md*α*6* (Gao et al., 2007c), as in the case Pearl-Sel strain of *Plutella xylostella* as described in Chapter 3. Additionally, spinosad resistance in the Spino-Sel strain of *Plutella xylostella* is likely due to detoxification by P450s and esterases (Sayyed et al., 2008). Therefore, the mechanism of spinosad resistance may not be similar in all cases (Scott, 2008). Assessing the role of the α*6* subunit in spinosad resistant strains would assist in providing a more complete understanding of spinosad resistance.

I would use the Pearl-Sel strain to investigate the role of *Pxyl*α*6* in other strains of *P. xylostella*. The CH_1 strain of *P. xylostella* has a similar pattern of inheritance for spinosad resistance as Pearl-Sel (Sayyed et al., 2004). If the spinosad resistance locus in CH_1 is the same as in Pearl-Sel, the resulting offspring from reciprocal crosses of the Pearl-Sel and CH_1 strains should show high levels of spinosad resistance. Additionally, this genetic complementation test with the Pearl-Sel strain can be used to determine the mechanism of spinosad resistance in field collected insects, which exist in many populations (Zhao et al., 2002, Sayyed et al., 2004, Zhao et al., 2006).

A diagnostic PCR assay could be used to assess the occurrence of truncated transcripts in spinosad resistant populations. This technique has a major advantage over genetic complementation tests because cDNA from pools of insects can be used to rapidly determine truncated transcripts. I would use primers that amplify the open reading frame of *Pxylα6*, then clone and sequence the PCR products. This is the preferred method because there are more than 11 *Pxylα6* transcripts that contain premature stop codons (Figure 3.3).

8.2 Exploring the Function of Transcriptional Diversity in *Tribolium castaneum*

I identified a diverse array of nAChR transcripts from *T. castaneum* (Table 4.2). However, there has been very little work that has identified a role for these diverse transcripts. There were eight transcripts of *Tcasα6* that contained premature stop codons due to cassette exon use (Figure 4.2). It would be of great value to examine if these transcripts with premature stop codons could provide the genetic basis for the selection for spinosad resistance as in the case of *P. xylostella* (Chapter 3).

8.3 Improving *Dα6* RNAi in *Drosophila melanogaster*

The Gal4-UAS system of *D. melanogaster* did not reduce the expression of *Dα6* to extremely low levels. There are a few technical improvements that could have been used to reduce *Dα6* expression further. The use of a double homozygote male for the *Dα6*dsRNA-transgene on the second and third chromosome may further reduce the expression of *Dα6*. This strategy has been successfully used to significantly reduce the expression of *dAdar* in *D. melanogaster* (Jepson and Reenan, 2009). This method, while tedious and time consuming, would be achievable with my current skills and infrastructure.

I hypothesize that the difference in the expression levels of *Dα6, Dicer1* and *Dicer2* may provide inadequate conditions for RNAi for *Dα6* in the brain and abdominothoracic ganglion (Chapter 5.3 and Figure 5.2). Therefore a method to increase the expression of *Dicer1* and *Dicer2* would substantiate this hypothesis. I would create a transgenic fly that carried a construct for the expression of Gal4 under the promoter for *Dα6*. This would allow for highly localized expression of Gal4 in tissues that also express high levels of *Dα6*. Flies with this construct would be crossed to a strain that contains a transgenic construct in which UAS controls the expression of either *Dicer1* or *Dicer2*. The resulting offspring would express high levels of *Dicer* in tissues with high levels of *Dα6* expression. These larvae with high levels of *Dicer* expression would be fed on media containing *Dα6* dsRNA encapsulated in cationic liposomes to activate RNAi against *Dα6* (Whyard et al., 2009). The emerging adults would be bioassayed with spinosad and *Dα6* expression would be quantified by qPCR as in Chapter 5.2.4 and Chapter 5.2.2, respectively. I expect that if *Dα6* expression is reduced to low enough levels (i.e. near zero), I should see an increase in spinosad insensitivity. This strategy would require outside expert assistance in developing strains with the transgenic constructs mentioned above.

8.4 Specific Reduction of RNA Editing in *Dα6* and *Dβ1* Expressing Tissues

The strategy used to assess the role of editing on insecticide sensitivity focused on a tissue specific role of editing. The contribution of RNA editing of *Dα6* and *Dβ1* on sensitivity to spinosad and imidacloprid is logical based on previous work (Perry et al., 2007). However, the influence of the editing on these particular nAChR subunits may be addressed more accurately using drivers that are specific for those genes. For example, I would create two strains of transgenic fly that expresses Gal4 under the control of either the *Dα6* or *Dβ1* promoter to reduce

editing in all tissues that express those nAChR subunits. These flies will be crossed to the dAdar strain to reduce RNA editing on those tissues expressing *Dα6* or *Dβ1*.This will help to more accurately define the contribution of RNA editing of those subunits on insecticide sensitivity.

8.5 Tissue Distribution of *Dα6*

Expression of *Dα6* is largely restricted to the nervous system (Chintapalli et al., 2007). However, the resolution of tissue distribution is murky and based on general body regions and tissues. The reduction of editing in cholinergic neurons, muscle and glia suggests the pattern of *Dα6* expression is much more complex (Chapter 6.3.1). Creation of the *Dα6*-Gal4 strain as mentioned in Chapter 8.4 would allow for a more detailed investigation of the tissue distribution of *Dα6* expression throughout the course of development. The *Dα6*-Gal4 strain could be easily crossed to a strain containing a construct for green fluorescent protein (GFP) under control of UAS. Progeny of this cross would express GFP in all tissues that also express *Dα6* which would easily allow for fluorescent imaging of *Dα6* expression. Colocalization of GFP expression with antibodies with fluorescent probes specific for tissues of interest (i.e. α-choline acetyltransferase to label cholinergic neurons) would provide an extremely informative map of *Dα6* expression.

8.6 Effect of RNA-Editing on Sulfoxaflor Sensitivity

Sulfoxaflor is a novel insecticide that interacts as a nAChR super-agonist, but at a site on the receptor composed of subunits distinct from those that bind neonicotinoids (Watson et al., 2011). Performing sulfoxaflor bioassays against editing deficient flies as described in Chapter 6, I can evaluate the role of editing on the interaction of sulfoxaflor with the nAChR. This is a worthwhile investigation because sulfoxaflor's unique interaction at the nAChR.

8.7 Heterologous Expression of Edited nAChR Subunits

RNA-editing affects the sensitivity of spinosad and imidacloprid *in vivo*. However, the effect of RNA editing on nAChR function is very limited (Yao et al., 2009). I propose to investigate the effect of A-to-I RNA editing on heterologously expressed nAChRs containing edited subunits (i.e. *Dα5, Dα6, Dβ1* and *Dβ2* (Sattelle et al., 2005)) on the sensitivity of spinosad and imidacloprid. I would utilize the two-electrode voltage clamp recording method to document changes in receptor physiology.

Edited receptors will be created using site-directed mutagenesis to create clones with each of the editing sites that have been previously identified for the *Dα5, Dα6, Dβ1* and *Dβ2* subunits (Grauso et al., 2002, Sattelle et al., 2005). The cRNA of edited *D. melanogaster* nAChR subunits will be injected into *Xenopus* oocytes along with cRNA of the rat *β2* subunit. Two-electrode voltage clamp protocols will be performed as previously described (Liu et al., 2006). Acetylcholine, spinosad, and imidacloprid will be applied via bath perfusion across a range of doses that will elicit a stable plateau response to the low dose through a dose that causes a fall off after peak response. The maximum current (I_{max}) and agonist concentration for 50% maximum response (EC_{50}) will be calculated. Dose response curves will be fitted with the Hill equation from data of four or more independent experiments obtained from different oocytes.

I expect editing of *Dα6* to cause changes in the receptor physiology when exposed to spinosad. Specifically, spinosad potency and efficacy would be reduced at edited receptors. Bioassay data indicates that reduced editing makes *Drosophila melanogaster* more sensitive to spinosad (Chapter 6.3.2). Bioassays have shown that *Dα6* null mutants are much less sensitive to spinosad and neurophysiology indicates the ventral ganglion is insensitive to spinosad (Chapter 6.3.2 (Watson et al., 2010, Perry et al., 2007)). I do not expect editing of *Dα6* to change the

response to imidacloprid because it has been demonstrated that imidacloprid does not act on receptors containing *Dα6* (Orr et al., 2009, Watson et al., 2010). Therefore, this research will establish the connection between previous work that showed that A-to-I RNA editing and receptors containing the *Dα6* subunit are involved in the sensitivity of spinosad.

I anticipate receptors containing edited versions of *Dβ1* will reduce the potency and efficacy of imidacloprid based on work reported here and elsewhere (Chapter 6 and (Yao et al., 2009). I expect similar results with edited versions of *Dβ2*, because mutations in *Dβ2* have been implicated with reduced mortality due to imidacloprid (Perry et al., 2008). It is uncertain what effect editing of the *Dα5* subunit will have on the potency and efficacy of either spinosad or imidacloprid because there have been no reports on its involvement as either the target site or resistance mechanism for spinosad or imidacloprid. However, consistent with prior hypotheses concerning the *Dα6* or *Dβ1* subunits, it is possible that editing will reduce the potency and/or efficacy to spinosad, imidacloprid or both.

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